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PhD Thesis

THE ROLE OF T LYMPHOCYTES AND MACROPHAGES IN EXPERIMENTAL MODELS OF ALLO- AND XENOGRAFT REJECTION

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CONTENT

PREFACE

An early attempt to replace the defected tissues by healthy ones date back thousands of years, at least. It has been only a few decades, however, since the first organ transplantation was successfully accomplished. Today, transplantation procedures may look as a daily routine to nonexperts, and in a sense, they really are. Such a rapid progress has been achieved mainly due to a major breakthrough in the field of immunology and discovery of effective immunosuppressive drugs; i. e., substances capable of suppressing the immune system response. Rejection of organs (tissues) by recipient's immune system, termed immunological graft rejection, remains a major obstacle in further development of clinical transplantations. Critical shortage of allotransplants (donation of organs or tissues between individuals of the same species) enhanced an interest in the use of tissue from other species (xenografts) as a potential source of donor material in clinical medicine.

The future goals of transplantation are the prevention of the rejection by therapies that should reduce or eliminate the need for long-term use of immunosuppressive drugs, the induction of a graft-specific tolerance, and the development of xenografts as a practical solution to organ availability.

ABBREVIATIONS

AC	anterior chamber
ACAID	anterior chamber-associated immunodeviation
APC	antigen-presenting cell
CoS	costimulator
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein-4
DC	dendritic cell
DTH	delayed-type of hypersensitivity
FasL	Fas ligand
HO	heme oxygenase
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
IL	interleukin
KO	knock out
LC	Langerhans cell
mAb	monoclonal antibody
mH	minor histocompatibility
MHC	major histocompatibility complex
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
eNOS	endothelial nitric oxide synthase
Tc	cytotoxic T cell
TcR	T cell receptor
TGF-β	transforming growth factor beta
Th	T helper cell
TNF-α	tumor necrosis factor alfa
TRAIL	ligand TNF-related apoptosis-inducing
WT	wild type
XNA	xenoreactive natural antibodies

LITERATURE REVIEW

1 INTRODUCTION

During the past quarter century, transplantation immunology has established itself as a scientific discipline to study the mechanisms by which recipient rejects or accepts a transplant from a different donor. Nevertheless, most laws of transplantation immunology had already been defined during the first two decades of the 20th century. Indeed, the field of transplant immunology had undergone dramatic expansion during the past few decades (Fig. 1A). The modern era of human allotransplantation started in the early 1960's with successful kidney transplant. At the same time there were also several clinical attempts with vascularized xenografts from primates (Reemtsma 1966).

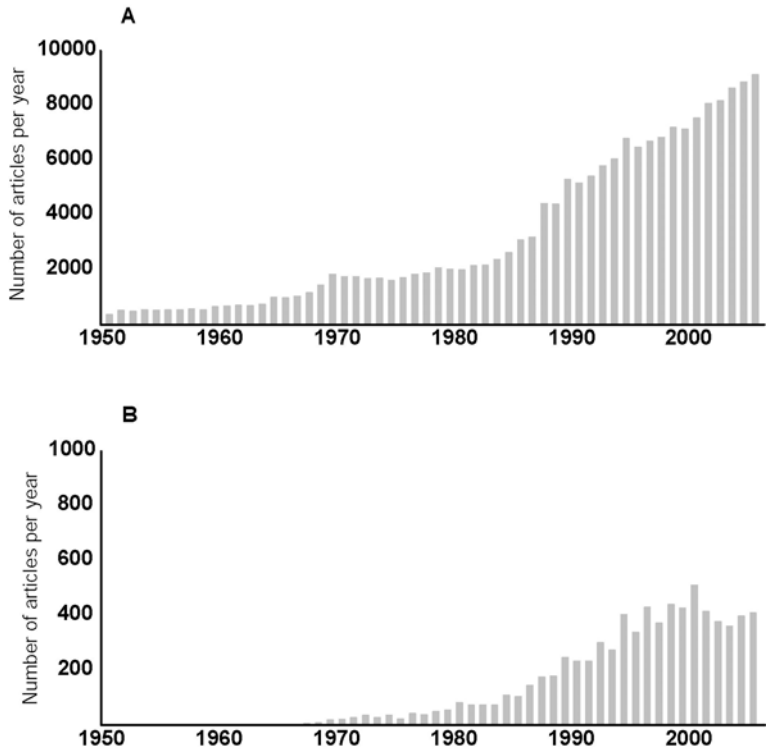

Fig. 1 Number of articles reported from the year 1950 to 2005.

Articles were counting using keywords graft and/or transplantation (A) and xenograft and/or xenotransplantation (B) in the title or full text. Data were compiled using web page http://highwire.stanford.edu

The increasing number of potential recipients lead to an organ deficit and there was renewed interest in xenotransplantation in the mid-1990s (Fig. 1B). Present research of xenotransplantation is currently focused on critical areas that include immunological barriers, physiological function, infectious disease risks, and pivotal ethical issues to make xenotransplants safe and effective for human beings.

In the 1960s, it was also recognized that macrophages and T lymphocytes are the predominant cell types infiltrating acutely rejecting allografts, in response that resembled delayed-type hypersensitivy (Brent et al. 1958). Since than, our understanding of the central importance of these cells in transplantation immunology has expanded immensely.

This thesis concentrates mainly on the role of macrophages and T lymphocytes and mechanisms known to be involved in the process of acute rejection of allo- as well as xenografts. Functioning of these cells in the rejection process was determined by monitoring changes in cytokines production, enzyme activity and release of their effector molecules in the site of rejected graft.

2 EXPERIMENTAL MODELS OF TRANSPLANTATION

In clinical medicine success of graft survival is still increasing due to the usage of new immunotherapeutic methods developed in experimental models. The frequently used experimental models of transplantation can be divided into:

- Organ (heart, lung, kidney)
- Solid tissue (cornea, skin)
- Cell (stem cells, spleen cells, pancreatic islet cells)

Transplantation of skin and cornea in rodents is frequently used in immunology research. Experimental corneal transplantations are interesting thanks the immune privilege status of the eye, as discussed in following sections.

Skin transplantation

One of the frequently used models in experimental transplantology is skin grafting that has been known for many centuries. The first temporary skin graft was discussed in a medical journal in 1881 (http://www.med.umich.edu/trans/transweb/reference/timeline/881.htm). The method of experimental skin transplantation used today is in general similar to that developed during pioneering studies by Sir Peter Medawar and his colleagues (Billingham and Medawar, 1951) during the 1960s. Skin transplant rejection is a good indicator of immune response and may serve as a sensitive test of the treatment effects. An average time of skin allograft survival varies from 6 to 10 days (Hilgert et al. 1983, Rosenberg et al. 1987, Goss et al. 1993, Ming et al. 2003) similarly to xenograft survival (Krieger et al. 1997). Skin transplantation, both allograft and xenograft, is primarily studied as a model of acute rejection. One reason for this particularly strong reaction can be associated with skin specific features that are probably related to the superficial location of skin and the fact, that the skin is continuously exposed to antigens. The cells that may play an important role in skin transplantation are the Langerhans cells (LC), the major antigen-presenting cells (APC) in the skin.

Corneal transplantation

Corneal transplantation, which is also known as penetrating keratoplasty, in striking contrast to the failure rate of the other grafts, became one of the most successful forms of tissue allotransplantation. In uncomplicated cases, the rejection rate for corneal

transplants is approximately 10% in the first year in human (The Collaborative Corneal Transplantation Studies Research Group, 1992).

Corneal transplantation procedures were developed during the 19th century when almost all donor corneas used for grafting were xenografts. The modern era of corneal transplantation begun in the 1950s due to the improvements in surgical techniques and progress in understanding of immunology and pathophysiology of corneal grafts. The extraordinary success of corneal transplantation, which can be achieved in other grafts only with profound systemic immune suppression, has been related to various features of the cornea and ocular microenvironment that together account for its immune-privilege status of anterior chamber (AC).

Majority of our information about the rejection mechanisms derives from animal models of transplantation. The degree of immune privilege of corneal allografts has been defined and quantified primarily on rodent models of keratoplasty. However, there are some problems with data obtained from animal models and their application to the human setting. In addition, immunological rejection of corneas in mice and rats is more aggressive than in human, with rejection occurring on relatively short timescales (George and Larkin 2004). For example, major and minor histoincompatible corneal grafts from B10 mice placed in normal eyes of BALB/c mice are rejected within 8 weeks in approximately 50% of cases (Sonoda et al. 1992). Moreover, human recipients of corneal grafts are permanently treated with immunosuppressive drugs from the first day of transplantation.

Certainly, there is also importance of experimental model of corneal transplantation for clinical medicine. Experimental corneal and skin grafting offers a good system to test different forms of therapy, because of the simple anatomy and ability to directly visualize the transplanted tissue and any rejection response.

Immune privileged status of the eye

The cornea forms the outer surface of the eyeball together with the sclera and also serves as a part of the ocular biodefense system. The features of normal uninflamed cornea and ocular microenvironment associated with its immune privilege status can be divided into three groups: anatomical, physiological and immunological. From the anatomical point of view the cornea is one of the few avascular tissues in the body and is devoid of lymphatic and blood vessels. Thus, cornea or corneal graft respectively is isolated from both the afferent and efferent arms of the immune response (Niederkorn 1999, Rocha et al. 1998). The immunological (Fig. 2) and physiological factors related to the immune privilege status of uninflamed cornea and ocular microenvironment include:

- The center of corneal epithelium is endowed with a population of major histocompatibility complex (MHC) class II- CD80- CD86- CD11b- CD3- LCs (Niederkorn and Peeler 1988; Hamrah et al. 2002, 2003).
- Decreasing density of stromal dendritic cells (DC) from the limbus toward the center, which contains exclusively MHC class II- CD80- CD86- DCs (Hamrah et al. 2003).
- Secretion of immunosuppressive transforming growth factor-β (TGF-β) by corneal epithelial and endothelial cells that inhibits T-cell activation and proliferation (Qian and Dana 2001) and which has a profound capacity to suppress the stimulatory role of LCs and to downregulate MHC class II expression (Hamrah et al. 2002).
- Constitutively expression of immunomodulatory molecules on cell membranes of corneal epithelium and endothelium cells: Fas ligand (FasL, CD95) and TNF-related apoptosis-inducing ligand (TRAIL), members of the TNF superfamily, that play a pivotal role in protecting the eye from cell-mediated damage by selective apoptotic activity (Griffith et al. 1995, Wilson et al. 1996, Lee et al. 2002, Niederkorn 2004).

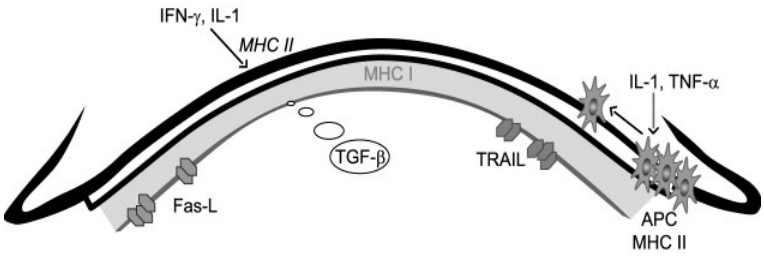

Fig. 2 Immunology of cornea.

Corneal epithelial cells, keratocytes and endothelial cells express primarily MHC I antigens. Expression of FasL and TRAIL and high concentration of TGF-β protect the cornea from cell-mediated damage. On the other hand, cytokines like IFN-γ and IL-1 stimulate epithelial cells to express MHC II molecules. Immature APCs can be stimulated by pro-inflammatory cytokines (IL-1, TNF - secreted by corneal endothelium) to migrate centripetally (modified according to Wilson et al. 1994).

Due to these features, allogeneic tissue implanted into the AC survives for prolonged intervals compared with tissues implanted subcutaneously or at other conventional non-immunoprivileged body sites (Streilein 1993). This hyporeactivity of AC results in a selective and adoptively transferable suppression of antigen-specific delayed-type of hypersensitivity (DTH), in the periphery known as a anterior chamber-associated immune deviation (ACAID) (Niederkorn 1990, 1999; Streilein 1993, 1999). However, immune privilege is a dynamic and not absolute state. Not all orthotopic corneal allografts

succeed, in humans or in experimental animals. Under circumstances promoting inflammation this privilege can be lost. One such setting is neovascularization, which can significantly increase the risk of graft rejection, e.g. by inducing vascularization in the recipient bed with stitches (Dana et al. 1996). Grafts placed into prevascularized 'high-risk' beds exhibit rejection rates of 50-90 % in human, even with a local and systemic immune suppression (Mader and Stulting 1991, Dana and Streilein 1996, Ksander et al. 1996) and 100 % in untreated experimental recipients (Vitova et al. 2004).

The special properties of cornea and ocular microenvironment allow corneal allograft prolonged survival. The orthotopic corneal xenografts are rejected within 7-10 days after transplantation, similarly to other tissue xenografts, which are rejected acutely in a process of cellular rejection (Qian and Dana 2001).

3 GRAFT REJECTION

In various experimental animal models and clinical transplantation, activated T cells, macrophages or specific antibodies are capable of mediating graft rejection via different mechanisms. For historical reason, the classification of graft rejection is based on histopathology and/or the time course of rejection after transplantation rather than immune effector mechanisms.

Rejection of allograft

Based on the experience, the histopathologic patterns of allograft rejection are called hyperacute, acute, and chronic. Hyperacute rejection begins within minutes to hours and is mediated by preexisting antibodies in the host circulation that bind to donor endothelial antigens. Complement activation leads to endothelial injury and exposure of subendothelial basement membrane proteins that activate platelets. These processes contribute to thrombosis and vascular occlusion, and the grafted tissue undergoes irreversible ischemic damage.

Process of injury mediated by T cells, macrophages and antibodies that usually begins after the first week of transplantation is typical for acute rejection. T cells play a central role because the activated $CD4^+$ T cells secrete cytokines that recruit and activate inflammatory cells and induce DTH-like reaction. In turn, such reaction can cause direct lysis of graft cells and necrosis of tissue.

Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over the prolonged period. This type of rejection can develop in any vascularized organs within a few months to years after transplantation. The fibrosis of chronic rejection may represent wound healing after the cellular necrosis of acute rejection, or result from activated macrophages, which secrete factors stimulating fibroblast proliferation (Abbas et al. 2000).

Rejection of xenograft

Xenogeneic donors can be divided into discordant and concordant. Species combination is considered discordant, when recipients pre-form xenoreactive natural antibodies (XNA) that are reactive with the carbohydrate determinates expressed by the cells of some mammals (Galili et al. 1987). In contrast, in concordant species combination XNA are found at low titer or absent.

The presence of natural antibodies roughly correlates with the phylogenic disparity between species. It is thought that XNA are responsible for the initiation of hyperacute rejection by which discordant vascularized xenografts are rapidly (in a few minutes or hours) destroyed. The same mechanism has been seen in hyperacute allograft rejection (Platt et al.1990).

Survival time can be prolonged for a few days if complement is inhibited or antibodies removed. In this case the graft being ultimately damaged by a form of acute vascular rejection, also called delayed xenograft rejection. This type of rejection includes a non-T cell-mediated cellular response as well as a T cell-mediated response. The process is characterized by intravascular thrombosis and fibrinoid necrosis of vessel walls. Mechanisms are not completely understood but rejection is accompanied by cytokine-mediated endothelial activation, and by presence of natural killer (NK) cells, macrophages and eosinophiles (Bach et al. 1996, Auchincloss et al. 1998, Platt 2002). In a process of cellular rejection, the xenograft is rejected by T cell-mediated immune response within a few days, the dominating cells infiltrating graft, apart from T cells, are also macrophages and NK cells (Lin et al. 1997). The mechanisms are similar to those that have been described for acute allograft rejection but they are more aggressive.

The most studies focuse on prevention of hyperacute or delayed xenograft rejection. Only limited number of studies is concentrated on chronic xenograft rejection. In the 1960's, this slow but progressive destructive process has been described in initially successful concordant (chimpanzee-to-human) xenografts, where XNA and complement are not involved primarily (Reemtsma 1966). Knechtle et al. (1987) were the first who achieved survival longer than 100 days in the concordant heart transplantation model. Therapy consisted of total lymphoid irradiation combined with cyclosporin (CsA). Within the same model of concordant aortic xenografts, it was observed that blood vessels had signs of vasculitis and accelerated arteriosclerosis, which occur extremely rapidly and are faster than in allogeneic grafts. It was also speculated that antibodies contribute to development of chronic rejection (Scheringa et al. 1996, Tanemura et al. 2000).

Immunosuppression - prevention and treatment of graft rejection

If the recipient of a graft has a fully functional immune system, transplantation mostly results in some form of rejection. Immunosuppression is the major strategy used for the prevention and management of transplant rejection in clinical practice as well as in the experimental models.

A possiblle way to reduce graft immunogenicity is to minimize alloantigen differences between the donor and recipient. Test for the presence of preformed antibodies against

MHC antigens of donor cells is called "cross-matching" and it is done for most types of organ graft, with the cornea being an exception.

Graft rejection may be also prevented by making the host tolerant to the antigens of the graft. It is presumed that the tolerance to a graft could involve the same mechanisms that are involved in tolerance to self-antigens (Abbas et al. 2000). Tolerance to grafts can be induced by classical procedures such as neonatal injection of allogeneic cells (Billingham et al. 1953, Hašek 1953). Prolonged graft survival was also achieved in combination with administration of immunosuppressive drugs (Mayumi et al. 1985), T cell depletion with monoclonal antibodies (mAb), or with non-depleting anti-T cell antibodies (Quin et al. 1989).

4 MECHANISMS OF GRAFT REJECTION

Multiple evidence indicating that graft rejection is a complex process involving mechanisms of immune responses, generated by contributions from both innate and adaptive immunity.

Innate immune response and graft rejection

Innate immunity is a primitive, highly conserved process by which signals, released by invading organisms or damaged tissues, are firstly recognized by macrophages, polymorphonuclears and NK cells (Matzinger 2002). The observations suggested that, similar to infectious models, the early phase of rejection consists predominantly of innate immune responses, whereas the late phases of rejection are enriched for components of adaptive immunity (Fox et al. 2001). Previous results of analyzing proinflammatory responses in graft recipients deficient in T and B lymphocytes have characterized a robust innate immune response that occurs during the first 24 h following transplantation (He et al. 2002). Innate response involved multiple proinflammatory molecules including chemokines, cytokines, and their receptors. On the model of xenotransplantation and, to a lesser extent, allotransplantation it has been demonstrated rapidly elicit innate response that precede the influx of T cells and occur even in their absence. Neutrophils appeared to be the first cells infiltrating graft that release chemoatractants, which recruit both monocytes and T cells, and initiate cell-mediated immunity (Mackay et al. 1999). The stronger stimulation of innate immunity by xenograft, compared with allografts, would be consistent with expression disparate non-polymorphic molecules, whereas allograft disparities are largely restricted to the polymorphic MHC proteins (Fox et al. 2001).

Graft rejection requires interaction between innate and adaptive immunity. Innate immunity serves as an important link between antigen-independent and antigen-dependent responses promote graft rejection (He et al. 2002, Moberg et al. 2005). Present studies are focused on role of innate immunity in antigen presentation, T cell priming and recruitment of T cells required for macrophage activation and their function in xenograft compared to the allograft rejection (Fox et al. 2001, Devos et al. 2005).

Graft recognition and adaptive immune responses

Immune responses are a major barrier to effective tissue transplantation, destroying grafted tissue by an adaptive immune response to its foreign proteins. These responses

can be mediated by different type of cells. Briefly, recognition itself can be further subdivided into discrete steps (Fig. 3), including afferent and efferent phase (Qian et al. 2001).

Afferent phase includes:

- Activation of APCs and their migration into the graft
- Processing of antigens
- Presentation of antigens in the context of MHC class II molecules to the T-cell receptor (TcR) of naive T cells in the draining lymph nodes, which together with adequate costimulation, results in T-cell activation

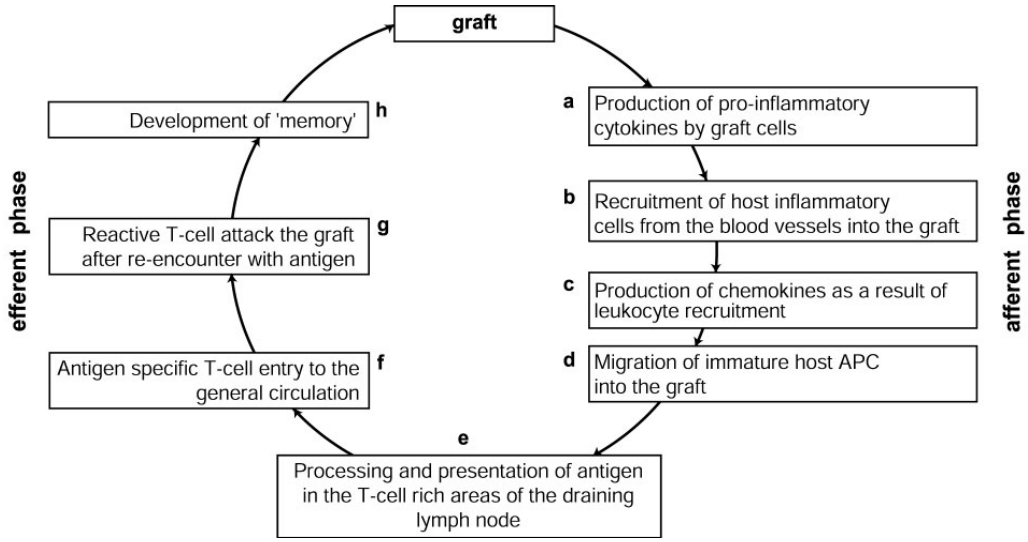

Fig. 3 Processes that lead to a cell-mediated immune response against graft.

This process consist of afferent phase (a, b, c, d, e) and efferent phase (f, g, h). (a) Production of pro-inflammatory cytokines in the graft as a result of grafting leads to (b) recruitment of host inflammatory cells from the blood vessels into the graft. (c) Leukocytes recruitment and chemokine production induce (d) migration of immature host APCs. Matured APCs leave the graft by entering afferent lymphatics to access draining lymph nodes. (e) After processing and antigen presentation in T-cell rich areas of lymph nodes (f) antigen specific T-cell entry to the general circulation. The final, effector phase of immune response involves the (g) targeting of the graft by reactive T-cell and (h) development of memory (modified according to Qian et al. 2001)

The expression or efferent phase of the response is synonymous with the process of attacking the graft or destructive response. This can be divided into steps consisting of:

- Entry of reactive T cells from lymphoid organs to the general circulation
- Delivery of these cells to the target tissue and re-encounter with antigen
- Development of 'memory', which might facilitate the expression of the immune response if there is repeated exposure to antigen.

5 ALLOTRANSPLANTATION

The immune response to antigens of allograft can be both cell mediated and humoral. In general, T cell – mediated immune reactions are more important for rejection of transplanted organs.

Alloantigen presentation and T cell activation (allorecognition)

Allograft rejection clearly represents a response to transplantation antigens, especially of polymorphic major (MHC class I and II) and minor histocompatibility (mH) complex. For T cell activation two signals are required (Lafferty and Cunningham 1975): alloantigen binding to the TcR as the first signal and as the second signal an inductive molecule or costimulator (CoS), expressed by a metabolically active APC. Cell surface molecules involved in this process are CD28, CTLA-4 (T-lymphocyte-associated protein-4) and CD154 expressed by T cells interacting with the CD80/CD86 and CD40 molecules expressed by activated APCs and B cells (Schwartz 1992). Naturally, also other accessory molecules expressed by APCs, have also important costimulatory function.

Alloantigens can trigger the activation of recipient T cells via two distinct mechanisms: direct (donor APC-dependent) and indirect (host APC-dependent) type of antigen presentation (Fig. 4). In the direct pathway, alloreactive $CD4^+$ T cells recognize intact allo-MHC II molecules on the surface of donor APCs (Sherman and Chattopadhyay 1993, Sayegh and Turka 1998, Illigens 2002). Both $CD4^+$ and $CD8^+$ T cells can be activated by direct pathway, because $CD8^+$ T cells can directly recognize MHC I molecules on donor cells. In the indirect pathway, $CD4^+$ T cells recognize donor-derived MHC peptides processed and presented by recipient APCs in the secondary lymphoid organs – lymph nodes and spleen. By indirect pathway of recognition mH molecules of donor cells can be also recognized after processing and presentation on recipient APCs.

Some studies have indicated that both direct and indirect alloresponses can contribute to the rejection of allograft. There is also ample evidence to indicate that direct responses can induce rapid and acute form of rejection, whereas indirect-type of alloreactivity may be associated with a slow process of late acute or chronic rejection, although the exact effector mechanisms remain unknown (Benichou et al. 1992, Fangmann et al. 1992, Waaga et al. 1998, Illigens et al. 2002).

The alloimmune response and the rejection process may vary with the type of tissue/organ transplanted. Some of the key factor that influenced the pathway of T-cell allorecognition after transplantation is the site of placement of the graft with regards to lymphatic drainage and vascularization (Illigens et al. 2002).

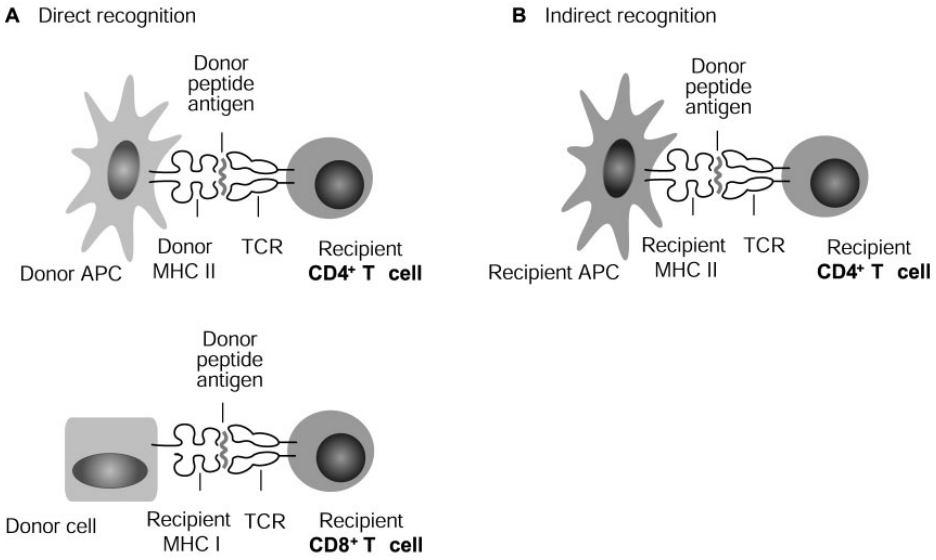

Fig. 4 Pathway of recognition in transplant rejection.

(a) Direct presentation. After transplantation, APCs from donor donor migrate out of the graft into recipient's $CD4^+$ T-cells. Similarly, recipient $CD8^+$ T cells can recognize directly donor peptide antigen present on MHC I of donor cells. (b) Indirect presentation. After transplantation, recipient APCs migrate into the graft, and take up and process the proteins from recipient cells. These donor peptide are presented on recipient MHC II molecules to recipient CD4+ T-cells in lymph nodes (modified according to Coates et al. 2002).

Role of T cells in allograft rejection

The rejection of skin and corneal grafts in untreated recipients, similarly to other fully allogeneic grafts, is primarily studied as a model of acute rejection. Predominant type of allorecognition in skin and vascularized grafts is direct recognition mediated by both T cell subsets $CD4^+$ and $CD8^+$ (Sherman and Chattopadhyay, 1993, Waaga et al. 1998, Illigens et al. 2002).

Corneal transplantation represents an interesting model, in that center of graft is naturally devoid of MHC class II^+ leukocytes and cannot theoretically elicit $CD4^+$ T cell-mediated direct alloresponse and therefore corneal grafts can predominantly trigger an indirect alloresponse focused on mH antigens (Streilein et al. 1979, Boisgérault et al. 2001). However, there is evidence than the direct and indirect pathway of sensitization may concur in corneal transplantation and the relative contribution of each pathway is based on multiple host and time-dependent factors, e.g. high-risk beds (Hamrah et al. 2003).

In the case of allotransplantation, $CD8^+$ T cells are able to recognize MHC class I antigens presented on allograft cells through the direct pathway. Even if direct CD8

response can induce graft destruction, the CD4 direct and indirect response is essential in the rejection. Probably from this reason, *in vivo* depletion of $CD4^+$ T cells with mAb anti-CD4 significantly enhanced survival of allografts, whereas treatment with anti-CD8 mAbs has no effect on the rejection rate (He et al. 1991, Han et al. 2000). The precise role of $CD8^+$ T cells is still controversial. Their cytolytic functions (Fas/FasL - mediated apoptosis and perforin/ granzyme release) and cytokine production are usually sufficient, but not always necessary, to ensure rejection. In the absence of CD4 alloresponse, $CD8^+$ T cells in direct fashion can mediate acute rejection for prolonged time period (Niederkorn et al. 2006). In turn, after depletion of $CD8^+$ T cells, remaining $CD4^+$ T cell response alone was sufficient to ensure the rejection process in normal timeframe (Boisgérault et al. 2001).

Allorejection as a cytokine-mediated process

The important role of $CD4^+$ T lymphocytes in allograft rejection has been demonstrated in a number of studies by using mAb anti-CD4 cells or gene knockout (KO) mice, which are genetically deficient in $CD4^+$ T lymphocytes (Pearson et al. 1993; Wen-Ruo et al. 1999; Han et al. 2000; Thiel et al. 2001). Direct and/or indirect allorecognition leads to activation of $CD4^+$ T helper (Th) cells, which can be further divided, based on their pattern of cytokine production at least into two functional subsets Th1 and Th2 (Fig. 5). Th1 cells secrete IL-2, interferon-γ (IFN-γ) and TNF-β and provide the necessary signals for the growth and maturation of macrophages and cytotoxic $CD8^+$ T lymphocytes (CTL) (Mosmann and Coffman 1987). Th2 cells secrete IL-4, IL-5, IL-10, IL-13 and are responsible for antibody production by B cells and activation of eosinophiles. Th1 and Th2 T cells can regulate each other. Thus, IFN-γ inhibits the development of Th2 cells, and IL-10 inhibits the secretion of cytokines produced by Th1 cells. This reciprocal regulation is so-called Th1/Th2 paradigm that provides a basis of understanding the mechanisms of rejection and tolerance in transplantation. Some results demonstrate that there is a hierarchy in the T-cell response associated with different types of allograft rejection: grafts with acute rejection predominantly induced the Th1 cytokines; those with delayed rejection is associated with a production of both Th1 and Th2 cytokines, whereas accepted grafts and grafts with long-term survival that they usually showed histological evidence of chronic rejection primarily expressed the Th2 cytokines (Nickerson et al. 1994, Le Moine et al. 1999, Mhoyan et al. 2003).

Cornea and skin are the tissues that are not primary vascularized, so hyperacute rejection, a rapidly occurring reaction involving antibody responses in the blood vessels does not occur (Gardner 1995, Qian and Dana 2001). It has also been described that corneal and skin allograft rejection is a $CD4^+$ Th1-cell-mediated process, demonstrated

by increase level of IL-2 and IFN-γ (Dollman et al. 1991, Satoru et al. 1998; Hargrave at al. 2000) and low or absent of production and expression of genes for IL-4 and IL-10 (Sano et al. 1998, Satoru et al. 1998).

Cytotoxic $CD8^+$ T lymphocytes can be also differentiated into T cytotoxic (Tc)1 and Tc2 population with cytokine profiles analogous to those seen in $CD4^+$ Th subpopulations: Tc1 producing high level of IFN-γ and Tc2 phenotype producing IL-4, IL-5, IL-10, IL-13 and low level of IFN-γ (Halverson et al. 1997, Delfs et al. 2001). After depletion of $CD4^+$ T cells, $CD8^+$ T cells can represent a source of cytokines required for graft rejection.

IFN-γ, produced by both $CD4^+$ and $CD8^+$ T cells, is considered one of the most important cytokine of Th1-mediated rejection (Hidalgo and Halloran 2002). IFN-γ is the proinflammatory cytokine with multiple effects on the immune system, upregulates MHC class II and costimulatory molecules on APCs, induces chemokine secretion, provides the stimulus for antibody switching, activates macrophages and has been implicated as a mediator of DTH (Paul and Seder 1994).

However, some studies have shown that allograft can be rapidly rejected in wild type (WT) as well as in IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) mice by $CD4^+$ T cells. After depletion of $CD8^+$ T cells, both WT and $IFN\text{-}\gamma^{-/-}$ mice rejected their allografts. This indicates that these mice share a common CD4-mediated, CD8-independent mechanism of rejection (Valujskikh and Heeger 2000, Bishop 2001).

Conversely, Th2 cytokines, especially IL-4 and IL-10, were described as cytokines with immunosuppressive properties and their production by graft infiltrating cells is associated with tolerance. There are several studies showing that exogenously administrating IL-4 and IL-10 before grafting leads to improve graft acceptance and survival (DeBruyne et al. 1998, Shinozaki et al. 1999, Mulligan et al. 2000; Quin et al. 2001; Miyamoto et al. 2005). Treatment of viral vector encoding IL-10 resulted in a significant reduction of neointimal proliferation and graft infiltration with macrophages and T and B lymphocytes. The mechanism underlying the protective effects of IL-10 in allografts also involved heme oxygenase 1 (HO-1) activity by which inflammatory cell infiltration is inhibited (Chen et al. 2005).

To determine the exact role of individual cytokines during rejection is complicated by their pleiotropic and redundant action. Antagonists against a single cytokine or knockout of one cytokine gene may not have functional effect, and other cytokines may compensate them.

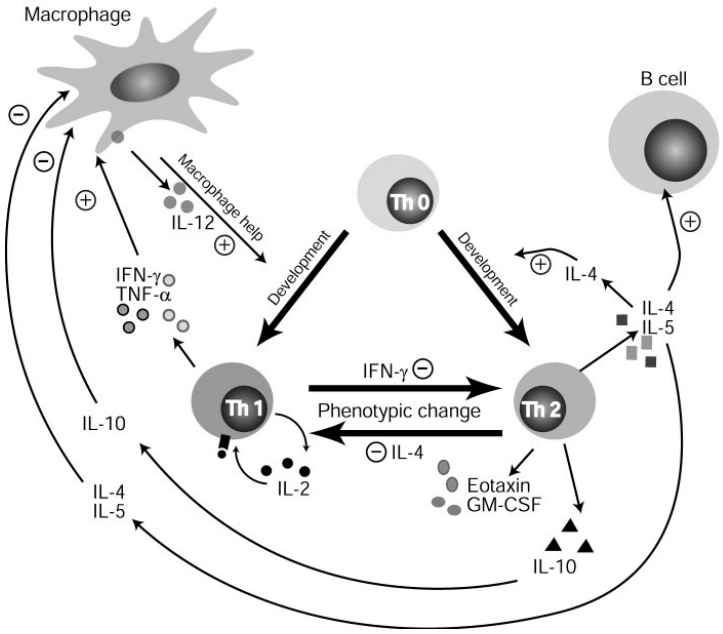

Fig. 5 A model of the interactions between polarized Th1 and Th2 responses.

Th1 and Th2 cells secrete a number of cytokines. By production of IFN-γ and IL-4 the lymphocyte subsets can regulate each other. Thus, IFN-γ inhibits the development of Th2 cells and IL-4 inhibits the differentiation and expansion of Th1 cells. Briefly, Th1 cytokines are associated with activation of macrophages and are often implicated in graft rejection. Whereas, Th2 cytokines are responsible for B cell activation and chronic graft rejection or tolerance (modified according to Harber at al. 2000).

6 XENOTRANSPLANTATION

The nature of cellular and humoral responses to xenogeneic tissues appears to differ from allograft immunity and is less understood. Cellular response during xenograft rejection is commonly more vigorous than during allograft rejection.

Xenorecognition

The intensity of immune response is determined by the interactions between T lymphocyte cell surface molecules and APCs. The T-cell subsets are characterized by CD4 and CD8 co-receptor molecules that interact with conserved region of class I, and class II MHC molecules, respectively. The interaction of these accessory/co-receptor molecules with their respective ligands can display species-specificity (Moses et al. 1992), and probably depends on the phylogenetic disparity between stimulating and responding species (Sachs and Bach 1990). Whether the recipient T-cells are capable of direct and/or indirect xenorecognition is still an open issue. Controversial results have been reported for both concordant and discordant species combination (Gill and Coulombe 1992, Gould and Auchinckoss 1999; Rogers et al. 2001, Tanemura et al. 2002). The above studies suggested the following scenario: xenografts are infiltrated by T cells and macrophages within first week after transplantation, if antibodies fail to cause graft rejection during this period. Many $CD4^+$ T-cell clones are likely to undergo activation within the xenograft by multiple immunogenic peptides that originate from xenoproteins, and are processed and presented by APCs. Unlike allograft rejection, the indirect pathway may dominate the T-cell response to xenografts. This may be partly due to incompatibilities of receptor/co-receptor interactions when the blockade of indirect recognition mediated by $CD4^+$ T cells leads to prolonged xenograft survival (Singh et al. 2004). From these findings the hypothesis was formed that a major pathway of $CD4^+$ T cells xenograft immunity is through indirect antigen presentation, and the role of direct pathway of antigen presentation is relatively less important (Chitilian et al. 1998, Shishido et al. 1998, Singh et al. 2004). The activation of $CD4^+$ T cells results in a local secretion of various cytokines, including IL-2. In turn, these cytokines induce differentiation of activated $CD8^+$ T cells into CTL that can kill xenogeneic target cells (Smyth et al. 1996). $CD8^+$ T cells activation also depends on ability of $CD8^+$ T cells to directly interact with xenogeneic MHC class I molecules. Some studies (Qian and Dana 2001, Yi et al. 2000) have demonstrated the capability of $CD8^+$ T cells to recognize both concordant and discordant xenogeneic MHC antigen *in vivo*. Ability to recognize MHC antigens in discordant xenograft combination is less effective than in concordant combination, and critically depends on B7/CD28 costimulation (Zhan et al. 2001). Although $CD4^+$ T cells

are major mediators in cellular rejection of xenograft, rejection still occurs in the absence of $CD4^+$ T cells, even if with delayed kinetics. Thus, in CD4-independent mechanisms of cellular rejection, activated $CD8^+$ T cells are able to destroy the transplanted tissue. While this mechanism is considerably less efficient than the $CD4^+$T cell-mediated one, it may be of greater importance when the development of chronic rejection is considered (Yi et al. 2000).

Th1/Th2 paradigm in xenograft rejection

Cellular type of rejection mediated by T cells of adaptive immune response takes place in xenograft models of transplantation. Two distinct subsets of $CD4^+$ T lymphocytes have been characterized by their cytokine profiles Th1 and Th2 as it was mentioned above. Unlike allografts, the pattern of cytokine gene expression and production in response to xenografts does not seem to be clear. Majority of studies demonstrated that xenoreactive cellular response in acutely rejected xenograft is associated with production of Th1 and Th2 cytokines simultaneously (Morris et al. 1995, Simeonovic et al. 1999 Kishimoto et al. 2000 Dujovny et al. 2002 Tanaka et al. 2005). Singh and Shirwan (2001) in their studies hypothesized that the indirect recognition pathway may specifically generate a Th2 response that contributes to acute xenograft rejection by regulating the humoral response. Their results showed that rejection of concordant xenograft is mediated by predominant production of Th2 cytokines and IFN-γ with low or absent production of IL-2 (Singh and Shirwan, 2001, Singh et al. 2003). The lack of IL-2 in the presence of high level of IFN-γ may suggest that this cytokine is expressed not by the classical Th1 T cells but by the cells of innate immunity, such as NK cells (Patselas et al. 1995). A Th2 response represented by cytokines IL-4 and IL-10 was detected in rejected grafts and draining lymph nodes, but the IL-10 transcript was detected at earlier time points, before increasing mRNA level of IL-4. This results in speculation that the main source of IL-10 can be predominantly the highly activated macrophages as one of the primary cells infiltrating graft (Krook et al. 2002). Inhibition of acute xenograft rejection was associated with decreased cytokine production in the graft, both Th1 and Th2 (Kishimoto et al. 2000). A different role for cytokine in concordant and discordant transplantation indicating local delivery of IL-10 and /or TGF-β into graft where they enhanced immune response and resulted in shorten xenograft survival of rat islet in mouse recipient in contrast to prolongation of graft survival in discordant species combination (canine-to-rat) (Deng et al.1997). On the other hand, islet xenograft rejection was also prevented by simultaneous but not single inhibition of IFN-γ receptor, TNF-α, and IL-2 (Benda et al. 2000).

7 MACROPHAGE-MEDIATED GRAFT REJECTION

Macrophages belong to the predominant cells infiltrating allo- and xenografts at time of rejection (Xia et al. 2000, Slegers et al. 2003, 2004, Axel et al. 2005). They are multifunctional cells participate on both innate as well as adaptive immune response during rejection (Slegers et al. 2004). During the innate response, macrophages promote inflammation by releasing TNF-α and IL-1. As participants in adaptive immune response they present antigen to primed T cells, thus they are thought to be one of the principal APCs (Slegers et al. 2003, 2004). Moreover, macrophages are able to express essential costimulatory molecules, adhesion molecules, and release chemokines and cytokines including IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18 and TGF-β (Wyburn et al. 2005). For that reason macrophage depletion became one of the way to find their particular role during rejection. The one of more widely used 'macrophage suicide' technique consists of the administration of clodronate-containing liposomes that are selectively toxic for macrophages (Van Rooijen et al. 1997). Local depletion of macrophages correlated with diminished expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1) and β2-integrins (Slegers et al. 2003) in corneal allografts. Clodronate treatment also markedly altered the mRNA levels of cytokines, where IL-1, IL-2, IL-4, IL-6 IL-10, IFN-γ, TNF-β were strongly decreased (Torres et al. 1999). Elimination of macrophages from the graft side prolonged graft survival (Fox et al. 1998, Wu et al 2001). Corneal allografts survived more than 8 weeks without any other form of therapy (Slegers et al. 2003; 2004). Adiminstration of liposomes also markedly delayed infiltration of T cells and eosinophiles (Fox et al. 1998, Wu et al. 1999).

Further studies brought evidence that macrophages are the key cytotoxic cell population in process of graft rejection (Yamamoto et al. 1998, Střeštíková et al. 2003), which are able to reject graft in the presence or absence of other effector cells (Wallgren et al. 1995, Slegers et al. 2003). In addition, macrophages activated by Th1 type cytokines produced nitric oxide (NO), an important effector and regulatory molecule in various models of immune response (Fig. 6). NO is catalytically formed by several isoforms of NOS which converts L-arginine to NO and L-citrulin (Brüne et al. 1998). Neuronal and endothelial NOS (eNOS) generate NO constitutively as a signaling molecule (Mayer and Hemmens 1997, Brüne et al. 1998). It has been suggested that NO produced by macrophages in rejected graft is generated via inducible NOS (iNOS) that can produce large amounts of NO for days or longer (Brüne et al. 1998, Yamamoto et al. 1998). The role of NO as a cytotoxic effector molecule during allograft rejection has been well demonstrated when prolonged allograft survival was achieved by suppression of NO production by selective iNOS inhibition (Worral et al. 1995, Holáň et al. 2001, Střeštíková et al. 2003) or NO scavenging (Roza et al. 2000).

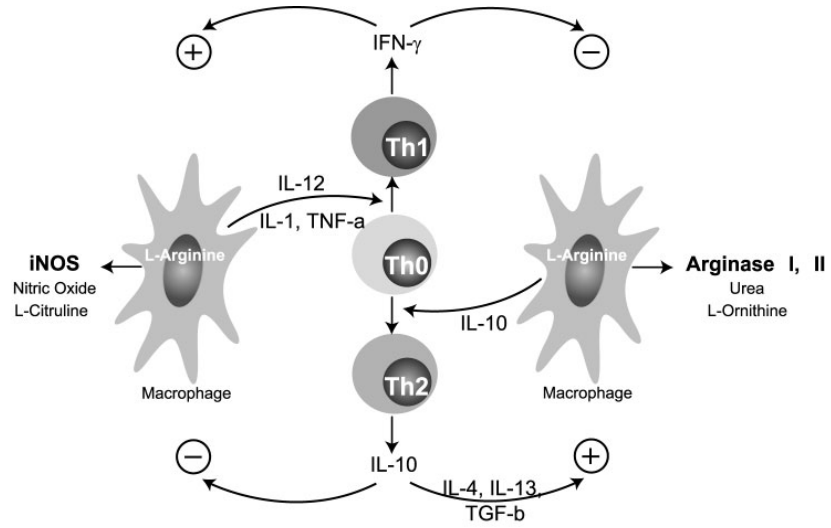

Fig. 6 A model of macrophage stimulation by Th1 or Th2 cytokines.

Macrophages activated by Th1 cytokines, especially by IFN-γ, produced NO that is catalytically formed by NO synthase, which converts L-arginine to L-citrulin and NO. In case of macrophage activation by Th2 cytokines (IL-4, IL-10, IL-13, TGF-β), enzyme arginase I and II is released. Products of arginase activity, which also requires a presence of L-arginine, are L-ornithine and urea. Simultaneously, two distinct macrophage populations regulate the function of T cells. Macrophages stimulated by Th1 or Th2 cytokines exert opposite effects on Th-cell development. Macrophages activated by Th1 cytokines generate IL-12 facilitate the development of Th1 cells, whereas macrophages activated by Th2 cytokines support the generation of Th2 cells.

On the contrary, macrophages stimulated by Th2 cytokines, the predominant cytokines of xenograft rejection, synthesize enzyme arginase. Two isoforms of arginase have been identified – arginase I and arginase II. They differ in cellular sublocalization and in tissue distribution. Several cells can express both isoforms such as mouse macrophages (Louis et al. 1998) or endothelial cells (Buga et al. 1996). Both isoforms convert L-arginine into L-ornithine and urea (Fig. 6). From this reason NO production may be reduced by arginase via depleting the common substrate in this cell types (Bronte et al. 2003). It has been confirmed in models such as Leishamia infection (Iniesta et al. 2001), human inflammatory disease (Bruch-Gerharz et al. 2003) or asthma (Morris et al. 2004). Thus, this regulatory mechanism of iNOS/arginase enzymes may regulate the effector mechanisms of transplantation reactions and can be important in xenograft rejection when the role of NO has not been well documented.

AIMS OF THESIS

General aims

On experimental models of skin and corneal allo- and xenotransplantation, we tried to explain immunological aspects participated in graft rejection. The main aim of this thesis was clarified the role of adaptive immune response, especially role of inflammatory cells infiltrating rejected graft - T cells and macrophages, their effector molecules, cytokines and enzymes (and their products) released during graft rejection.

Specific aims

- To investigate involvement and role of NO produced by macrophages in immune response to skin allografts in mice by using specific inhibitor of iNOS and demonstrate dependence of NO production on the presence of activated T cells.
- To show how treatment with mAb anti-CD4 or anti-CD8 affects the gene expression and production of Th1 and Th2 cytokines and iNOS in concordant corneal xenografts during acute rejection.
- To investigate the influence of local depletion of macrophages and/or T cells on rejection of corneal xenografts.
- To clarify the link between Th2 cytokine production and undetectable or only limited amount of NO in rejected skin xenograft.

PUBLICATIONS

The PhD thesis was elaborated on the basis of following studies:

- HOLÁŇ V, KRULOVÁ M, ZAJÍCOVÁ A, PINDJÁKOVÁ J. Nitric oxide as a regulatory and effector molecule in the immune system. Molecular Immunology 2001; 38: 989-995.
- PINDJÁKOVÁ J, VÍTOVÁ A, KRULOVÁ M, ZAJÍCOVÁ A, FILIPEC M, HOLÁŇ V. Corneal rat-to-mouse xenotransplantation and the effects of anti-CD4 or anti-CD8 treatment on cytokine and nitric oxide production. Transplant International 2005; 18: 854-862.
- HOLÁŇ V, PINDJÁKOVÁ J, ZAJÍCOVÁ A, KRULOVÁ M, ŽELEZNÁ B, MATOUŠEK P, SVOBODA P. The activity of inducible nitric oxide synthase in rejected skin xenografts is selectively inhibited by a factor produced by grafted tissue. Xenotransplantation 2005; 12: 227-234.
- HOLÁŇ V, PINDJÁKOVÁ J, KRULOVÁ M, NEUWIRTH A, FRIČ J, ZAJÍCOVÁ A. Production of nitric oxide during graft rejection is regulated by the Th1/Th2 balance, the arginase activity and L-arginine metabolism. Transplantation 2006; 81: 1708-1715.
- VÍTOVÁ A, PINDJÁKOVÁ J, JIRSOVÁ K, ZAJÍCOVÁ A, VAN ROOIJEN N, FILIPEC M, FORRESTER JV, HOLÁŇ V. Macrophages and CD4$^+$ T cells are both required for acute corneal xenograft rejection. In preparation.

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Nitric oxide as a regulatory and effector molecule in the immune system

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Abstract

Nitric oxide (NO) as a small ubiquitous molecule influencing a great variety of biological processes in the organism. Within the immune system, increased levels of NO were observed in various immunopathological situations, inflammatory reactions and during the response to transplantation and tumour antigens. It appears that NO can influence various facets of immune response. We studied involvement and the role of NO in immune response to skin allograft in mice. The production of NO at the site of graft rejection correlated well with the kinetic of rejection reaction and with the fate of the allograft. Graft infiltrating macrophages were identified as a principal cell population producing NO and the production of NO by macrophages was dependent on the presence of activated $CD4^+$ T cells. Survival of skin allografts was significantly prolonged by the treatment of graft recipients with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), a specific inhibitor of inducible NO synthase (iNOS). These results suggest a role for NO as the effector cytotoxic molecule involved in the graft rejection. Experiments in vitro demonstrated that NO, in addition to its effector function, acts as a modulator of cytokine production. Spleen cells stimulated with alloantigens in the presence of AMT or *S*-ethylisothiourea (EIT), an another selective iNOS inhibitor, produced considerably more interleukin (IL)-4 and IL-10 than the cells stimulated in the absence of iNOS inhibitors. The production of Th1 cytokines IL-2 and interferon (IFN)-γ was not enhanced by the inhibition of NO synthesis. The results altogether show that NO can act in transplantation reactions as an immunomodulator on cytokine production level and as an effector molecule involved in the graft destruction.

Keywords: Skin allografts; Macrophages; Nitric oxide; Cytokines; Immunoregulation

1. Introduction

Nitric oxide (NO) is a small molecule synthesised in the body from L-arginine by a family of enzymes, the nitric oxide synthases (NOS) (Knowles and Moncada, 1994). At least three isoforms of NOS have been identified (Forstermann et al., 1994). Two of them synthesize NO constitutively in a great variety of cells. The third isoform, called inducible NOS (iNOS), is activated by various stimuli and can produce a large amount of NO (Forstermann et al., 1994; Nathan and Xie, 1994).

One of the major sources of the inducibly produced NO are macrophages. Elevated levels of NO were found in infectious and inflammatory diseases (Miller and Grisham, 1995) autoimmune processes (Cross et al., 1994; Tilton et al., 1994,) and during the reaction to tumour and transplantation antigens (Lejeune et al., 1994; Ioannidis et al., 1995). NO has been shown as the effector cytotoxic molecule responsible for macrophage-mediated cytotoxicity (Hibbs et al., 1988). Overexpression of NO was suggested as the cause of down-regulation of systemic immunity observed in tumour bearers, and in animals with autoimmunity and inflammatory disorders (Cowden et al., 1998; Hegardt et al., 2000). Since macrophages can modulate cell proliferation of mitogen- or antigen-stimulated lymphocytes, NO was also considered as a possible immunoregulatory molecule in these systems (Mills, 1991; van der Veen et al., 2000).

In the transplantation immunity models, elevated concentrations of NO were detected during allograft rejection (Ioannidis et al., 1995; Worrall et al., 1996). Cytokine-activated macrophages infiltrating rejected allograft were suggested as a major source of NO (Langrehr et al., 1993). The importance of NO in transplantation reactions was supported by the observations that rejection reaction can be prevented by the treatment of graft recipients with iNOS inhibitors or NO scavenger (Worrall et al., 1995; Roza et al., 2000).

Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; Con A, concanavalin A; EIT, *S*-ethylisothiourea; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IFN, interferon; LPS, bacterial lipopolysaccharide; mAb, monoclonal antibody; NO, nitric oxide; PBS, phosphate bufferred saline

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GENERAL DISSCUSION

Rejection of allo- and xenograft is a process caused by specific response of the recipient immune system against transplanted tissue including a lot of different cell types and molecules. In our studies we focused on adaptive immune response and the role of T cells and macrophages in this process.

Nitric oxide and skin graft rejection

One of the effector molecules which are able to modulate immune response, is NO. This molecule is responsible for macrophage-mediated cytotoxicity due to its toxic effect on various cell types (Nathan 1995, Hibbs et al. 1998). Increased concentration of NO during allograft rejection has been described earlier (Ioannidis et al. 1995), but we showed on model of skin allotransplantation in mice that the presence of NO after transplantation correlates with graft rejection (publication 1). Furthermore, we demonstrated that production of NO by skin allograft infiltrating macrophages is depended on the presence of $CD4^+$ T cells. Treatment of graft recipients with CsA and mAb anti-Thy 1.2 or anti-CD4, but not anti-CD8, significantly decreased NO production in rejected allograft. We also found that specific inhibition of NO production by AMT a selective iNOS inhibitor together with mAb anti-CD4 not only prolonged allograft survival but also changed the Th1/Th2 cytokine production. Production of IL-4 and IL-10 was enhanced by inhibition of iNOS, while production of Th1 cytokines IL-2 and IFN-γ was decreased. Th2 cytokines production is generally associated with transplantation tolerance (Nickerson et al. 1994, Holáň 1998, Mhoyan et al. 2003). We suggested that the shift of immune response to Th2 direction by inhibition of iNOS might contribute to the immunosuppressive effects of iNOS inhibitors described in various models (Cross et al. 1994, Tilton et al. 1994, Worral et al. 1995). The involvement of NO in graft rejection has been supported by studies when graft rejection was delayed by treatment of graft recipient with selective iNOS inhibitors or by NO scavenger (Worral et al. 1995, Roza et al. 2000). Different results were achieved by using iNOS KO mice, when mice lacking iNOS normally rejected allograft (Wei et al. 1995, Casey et al. 1997). Probably, in these KO mice altered immunoregulatory mechanisms were developed.

Macrophages are considered as one of the main cell population infiltrating rejected allografts as well as xenografts (Xia et al. 2000, Slegers et al. 2003, 2004, Axel et al. 2005). Until now there was no evidence for production of NO or for the role of NO in

xenograft rejection. In our studies (publications 2 and 3) we used an experimental model of concordant (rat-to-mouse) skin xenotransplantation. We did not find any significant NO production by cultivated explants of rejected xenografts. Even in other species combinations (chicken-to-mouse, jird-to-mouse) undetectable or very low NO production was found (data not shown), while rejected mouse skin allografts produced a substantial amount of NO under the same conditions. Results of RT-PCR and western blot analysis respectively showed the expression of iNOS mRNA and accumulation of iNOS protein was comparable in allografts and xenografts. Low levels of citrulin, the co-product of NO synthesis, in supernatants of cultivated xenografts supported finding that the levels of NO in the supernatants were not scavenged during incubation of graft explants. We confirmed by using AMT, a selective inhibitor of iNOS, that NO does not play an important role in skin xenograft rejection. The treatment with AMT of xenograft recipients does not result in a prolongation of graft survival, whereas the same treatment enhanced survival of skin allografts (Holáň et al. 2001). Our findings are supported by the observations showed that pig proislets xenografts transplanted into iNOS$^{-/-}$ mice were rejected with normal kinetics (Simeonovic et al. 2002) and that depletion of macrophages in recipients prolonged discordant but not concordant xenograft survival (Axel et al. 2005). We also found, similarly to earlier published studies (Morris et al. 1995, Simeonovic et al. 1999, Kishimoto et al. 2000, Dujovny et al. 2002, Tanaka et al. 2005), that cultivated explants of xenografts produce considerably more Th2 cytokines IL-4 and IL-10 than explants of allografts, where production of Th1 cytokines predominated. It is well known that Th1 cytokine IFN-γ activates macrophages to produce NO by converting L-arginine to NO and citrulin. In addition, IL-4 and other Th2 cytokines increase macrophage arginine metabolism via arginase which produce ornithine and urea (Mills et al. 2000). Thus, production of NO depends on the availability of L-arginine, a substrate for iNOS which competes for L-arginine with arginase. Moreover the K_m for L-arginine is in the 2-20 mmol/L range for arginase compared with the 2-20 μmol/L range for various NOS isoenzymes that V_{max} of arginase is 1000-fold higher than for iNOS (Iyer et al. 1998, Morris 2002). Accordingly, an increased arginase activity in rejected xenografts may be a factor responsible for attenuation of NO production, as it has been described in experimental models of asthma (King et al. 2004) or leishmaniasis (Iniesta et al. 2001). To confirm this hypothesis we used anti-CD4 and anti-IL-4 treatment of xenograft recipients to eliminate production of Th2 cytokines or IL-4, respectively. The therapy decreased arginase activity and partially restored NO production in rejected xenografts. These experiments suggest that CD4$^+$ T cells and their Th2 cytokines are potent inducers of arginase and that through regulation of L-arginine metabolism they may be responsible for down-regulation of NO production.

Medium arginase activity and gene expression of mouse arginase I and II was also found in allografts. Mouse macrophages activated by Th2 cytokines are capable to express both arginase isoforms (Louis et al. 1998) and up-regulation of arginase I was also

observed in fibroblasts after wounding or Th2 stimulation (Witte et al. 2001). As for iNOS, arginase isoforms can be up-regulated by cytokines as well as by oxygen tension or trauma (Modolell et al. 1995, Luis et al. 1998, Ochoa et al. 2000). Using RT-PCR we detected the gene expression for mouse arginase I and II and rat arginase I in rejected xenografts. The up-regulation of rat arginase I in rejected xenografts can be attributed to the production of this isoform by fibroblast of rat skin. Rat arginase II was not detected in rejected xenografts, since rat macrophages are absent in the process of rejection. Expression of rat arginase I and mouse arginase I and II together with high production of Th2 cytokines can be responsible for a totally strong arginase activity in rejected xenografts in compare with allografts.

The role of NO as cytotoxic effector molecule in allograft rejection has been well documented. Our results presented in publication 1 using model of skin allograft rejection showed that NO is involved in the destruction of transplanted tissue. On the other hand we demonstrated in publications 2 and 3 that NO production during skin xenograft rejection is limited by L-arginine bioavalibity and that it depends on arginase activity and a local cytokine environment. Thus the role of NO as cytotoxic molecule participated on skin xenotransplantation reaction seems to be unclear.

Macrophages and CD4+ T cells in corneal xenograft rejection

Corneal allograft as well as xenograft is rejected acutely and acute rejection is dependent on the presence of $CD4^+$ T cells (Yamada et al. 1999, Tanaka et al. 2000). In our studies (publications 4 and 5), on a model of rat-to-mouse corneal xenotransplantation, we demonstrated a pattern of the expression of genes for cytokines IL-2, IFN-γ, IL-4, IL-10 and for mouse iNOS and rat eNOS molecules in rejected grafts. We compared the expression of these genes in xenografts from untreated mice, mice treated with mAb anti-CD4, anti-CD8 alone or in combination with local depletion of macrophages. Moreover, we investigated the effect of this therapy on corneal xenograft survival.

We showed that activated T lymphocytes produce, in site of rejected corneal xenograft, both Th1 and Th2 cytokines. We found that mice treated with mAb anti-CD8 rejected corneal xenografts with a similar kinetics as untreated recipients. Simultaneously the expression of genes for the tested cytokines IL-2, IFN-γ, IL-4 and IL-10 and their production were maintained. Corneal xenograft survival in mice treated with mAb anti-CD4 was significantly prolonged and was associated with the absence of cytokines IL-2, IL-4 and IL-10. In all rejected grafts of untreated or treated recipients production of IFN-γ was always detected. These results suggest that cytokines IL-2, IL-4 and IL-10 are largely produced by $CD4^+$ T cells and they are not necessary for prolongation of corneal

graft survival. On the contrary, the production of IFN-γ in rejected xenografts of anti-CD4 treated recipients confirmed that other cell types than $CD4^+$ T cells can produce IFN-γ. This observation is in agreement with findings of Higuchi et al. (2003) who showed that corneal xenografts that avoid acute rejection in $CD4^+$ T cells depleted mice are vulnerable to delayed rejection mediated by IFN-γ releasing $CD8^+$ T cells. On the other hand, depletion of $CD8^+$ T cells does not prolong xenograft survival and thus it suggests that $CD4^+$ T cells play a more important role than $CD8^+$ T cells in xenotransplantation reaction. IFN-γ is necessary for activation of macrophages to release NO, the main effector molecule responsible for macrophage-mediated cytotoxicity. The production of NO in rejected corneal xenografts has not been described. We found expression of the gene for iNOS and NO production in all rejected xenografts from untreated recipients or recipients treated with mAb anti-CD4 or anti-CD8. NO production can be considered as indirect evidence for the role of macrophages in corneal xenograft rejection and thus supports the findings of other studies showing macrophages as the predominant infiltrating cells in rejected xenografts (Wallgren at al. 1995, Wu et al. 1999, Yi et al. 2003). In our study, local depletion of macrophages with clodronate-LIP significantly prolonged corneal xenograft survival. Moreover, we found undetectable mouse iNOS gene expression in these rejected xenografts. In spite of that, low levels of NO production were observed in all cultures of cultivated grafts. Probably, expression of the gene for rat eNOS was responsible for this NO production. Nevertheless, all xenografts in clodronate-LIP-treated recipients were rejected within three weeks, in contrast to corneal allograft, where grafts survived more than eight weeks (Slegers et al. 2003; 2004). This may be explained by a participation of other cell types in rejection, such $CD8^+$ T cells, NK cells or complement-mediated mechanisms.

In our study combined depletion of macrophages and $CD4^+$ T cells enhanced survival of corneal xenografts, but the mean survival time of these xenografts was similar to that of anti-CD4 treated recipients. It suggests that effector mechanisms used by macrophages during xenograft rejection are not so effective than in allograft rejection. Moreover, NO production may be depressed by arginase activity by the same mechanisms as it was mentioned above in part of discussion about skin xenograft rejection.

Besides, treatment with clodronate-LIP also reduces corneal lymph and hemangiogenesis (Cursiefen et al. 2004). Postponed antigen presentation in draining lymph nodes may be another factor contributing to the delayed graft rejection in our model.

In summary, corneal xenograft rejection is associated with Th1 and Th2 cytokine production. Depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, reduces production of IL-2, IL-4 and IL-10 and markedly prolongs xenograft survival. However, rejection of corneal xenografts in untreated or treated recipients is associated with expression of genes for IFN-γ and iNOS. Depletion of macrophages also enhanced xenograft survival,

but combination of local depletion of macrophages and systemic depletion of $CD4^+$ T cells had no additional effect in comparison with depletion of $CD4^+$ T cells alone. These results suggest that macrophages and $CD4^+$ T cells play interdependent roles in acute corneal xenograft rejection. In addition, IFN-γ production appears to be integral component of rejection of corneal xenografts.

CONCLUSIONS

Our results on model of skin allo- and xenograft rejection shown that:

- NO, a cytotoxic effector molecule of macrophages, is involved in destruction of skin allografts
- Depletion of $CD4^+$ T cells significantly reduced NO production during allograft rejection
- Rejection of skin xenografts is associated with production of both Th1 and Th2 cytokines, in contrary to allografts, where production of Th1 cytokines predominates
- NO production during xenograft rejection is limited by L-arginine bioavailability and it depends on arginase activity

From experiments on model of rat-to-mouse corneal xenograft rejection we concluded:

- Rejection of corneal xenografts is accompanied with production of both Th1 and Th2 cytokines, together with expression of gene for iNOS and NO production
- Depletion of $CD4^+$ T cells, but not $CD8^+$, enhanced survival of xenografts and results in decreased production and expression of genes for IL-2, IL-4 and IL-10 in corneal xenograft explants
- Treatment of recipients with mAb anti-CD4 and anti-CD8 did not affect the production of IFN-γ and NO and their gene expression
- Depletion of macrophages by local administration of clodronate-liposomes prolongs corneal xenograft survival and was associated with undetectable expression of the gene for iNOS
- Macrophage depletion in combination with mAb anti-CD4 had no additional effect on prolongation of corneal xenograft survival in comparison with depletion of $CD4^+$ T cells alone

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