Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Doktorský studijní prográm v biomedicině
Studijní obor: Imunologie

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Genetic and molecular factors influencing the outcome of solid organ transplantation

Genetické a molekulární faktory ovlivňující výsledky transplantací solidních orgánů

Disertační práce

Vedoucí závěrečné práce/Školitel: Doc. MUDr. Antonij Slavčev

Praha, 2013

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Podpis

Abstract

Since its beginning, graft rejection remains the key problem of solid organ transplantation. This reaction of the recipient's immune system against mismatched antigens of the transplanted organ causes graft damage and consequently loss of its function. Rejection involves cellular (lymphocyte mediated) and humoral (antibody mediated) mechanisms.

Among the genetic factors which may have a prognostic value in rejection risk evaluation are the Human Leukocyte Antigens (HLA) genotype, the Killer Immunoglobuline-like Receptor (KIR) gene repertoir, cytokine and other gene polymorphisms. These factors could be screened for before transplantation to find the best possible combination of genetic characteristics of the donor and recipient and to reveal patients with "risky" genotypes, who may need more intensive immunosuppression and more careful post-transplant follow-up. Molecular factors, such as HLA and non-HLA antibodies, soluble CD30 molecule (sCD30), Hepatocyte Growth Factor (HGF) and other cytokines, measured before and/or after transplantation in the recipient's blood may be helpful for rejection risk estimation and may also be used as post-transplant rejection onset markers. In our study, we focused on some of the above mentioned factors.

We found that ethnicity plays a significant role in the distribution of both KIR genes and genes for KIR receptors' ligands in human genotype and that in the Czech population, the KIR repertoire is similar to other Caucasian populations. This should be taken into consideration when conducting clinical and comparative transplantational studies in different regions and when applying the results of other studies to the Czech population.

Our further finding was that the combined productions of HLA class I and II antibodies are the most unfavorable in kidney and heart transplantation, while pre-transplant MICA antibodies did not affect the incidence of either humoral or cellular rejection in heart transplant recipients. We also did not confirm that pre- and/or post-transplant sCD30 may be used as a stand-alone test for prediction of rejection in kidney transplant recipients. However, in patients with HLA antibodies, sCD30 levels are helpful for discriminating recipients who are at the highest and the lowest risk of rejection. Interestingly, concentrations of HGF before and after transplantation are not directly correlated with humoral and cellular rejection either in heart or in kidney graft recipients. Nonetheless, our data suggests that HGF might have an AMR-protective effect in kidney recipients with elevated pre-transplant sCD30; however, this capability needs further investigation.

Souhrn

Přes veškeré úsilí a dosavadní pokrok v oblasti orgánových transplantací a potransplantační imunosuprese, rejekce štěpu zůstává klíčovým problémem omezujícím přežití transplantovaných orgánů. Rejekce je reakci imunitního systému příjemce proti neshodným antigenům dárce, která vede k poškození tkaní a ztrátě normální funkce štěpu. Rejekce zahrnuje buněčné (lymphocyty zprostředkovaná rejekce) a humorální (protilátkami zprostředkovaná rejekce) mechanizmy.

Mezi genetické faktory, které mohou mít prognostickou hodnotu při hodnocení rizika rejekce, patří geny kódující lidské leukocytární antigeny (HLA), genetický systém Killer Immunoglobuline-like receptorů (KIR), cytokinové polymorphysmy a další geny. Předtransplantační vyšetření těchto faktorů může napomocí vyhledaní nejpříznivější kombinace genů mezi dárcem a příjemcem a odhalení pacientů s „rizikovým“ genotypem, které mohou potřebovat úpravu imunosupresivní léčby nebo obzvlášť pečlivé sledovaní v post-operačním období.

Molekulární faktory, jako například HLA-protilátky a non-HLA protilátky, solubilní molekula CD30, růstový faktor hepatocytů (HGF) a další cytokiny, změřené před a/nebo po transplantaci v krvi příjemce můžou jednak napomoci hodnocení rizika rejekce, jednak sloužit jako markéry probíhající rejekční epizody.

Tato práce je zaměřená na hodnocení některých ze zmíněných faktorů a závěry jsou následující.

Etnikum hraje velkou roli v distribuci KIR genů, genů KIR ligandů a v zastoupení jednotlivých KIR haplotypů. Toto se můsí brát do úvahy při extrapolaci údajů získaných z jiných etnických skupin a při provádění srovnávacích studií.

Naše studie prokázala, že kombinovaná produkce protilátek proti HLA I. a II. třídy je nejrizikovější pro vznik humorální rejekce jak u příjemců ledvin, tak u příjemců srdce, zatímco předtransplantační produkce MICA protilátek nemá výrazný vliv na výskyt humorální nebo buněčné rejekce. Koncentrace sCD30 před a/nebo po transplantaci není dostatečně spolehlivým samostatným prediktorem AMR a/nebo CR u příjemců ledvin. Avšak u pacientů s protilátkami proti HLA antigenům, měření koncentrace sCD30 napomáhá k detekci pacientů s největším a nejnižším rizikem rejekce. Jsme také zjistili, že hodnoty růstového faktoru hepatocytů (HGF) naměřené před a po transplantaci nejsou v přímé korelaci s výskytem akutní rejekce štěpu, ale výsledky naše práce naznačují že zvýšený HGF může mít protektivní efekt u pacientů se zvýšenou předtransplantační koncentraci sCD30.

Contents

Acknowledgments

This study was supported by the following grants from Internal Grant Agency of the Ministry of Health: NI/8981-3; NR/7859-3; NR/9403-3 and by the IKEM institutional grant MZO 00023001.

I would cordially like to thank my mentor, Assoc. Prof. Antonij Slavčev MD, PhD, for his great support, patience and criticism. My special thanks also go to all my colleagues from the Department of Immunogenetics of the Institute for Clinical and Experimental Medicine (IKEM) especially to Libor Kolesar, PhD., Mgr. Šárka Valhová and Helena Stehlíková for their help, cooperation and for having an inspiring atmosphere.

It is also my pleasure and honor to thank my beloved parents and my awesome sister for their kind support and help.

List of abbreviations

AECA – Anti-Endothelial Cells Antibodies

AMR – Antibody-mediated Rejection

BOS – Bronchiolitis Obliterans Syndrome

CDC – complement-dependent cytotoxicity

ChR – Chronic Rejection

CMV – Cytomegalovirus

CR – Cellular Rejection

EC – Endothelial Cells

FCXM – Flow Cytometry Cross-match

HLA – Human Leukocyte Antigen

KIR – Killer Immunoglobulin-like Receptor

MHC – Major histocompatibility complex

PCR – Polymerase Chain Reaction

SA – Single Antigen

TCAD – Transplant Coronary Artery Disease

Immunobiology of graft rejection

Since the very first experiments with solid organ and tissue transplantation, graft rejection has been the main obstacle for the successful graft survival and function. The concept of rejection and its' causes transformed from the Renaissance idea of a temperamental conflict between donor and recipient (Gasparo, 1597), to the current experimentally proven concept that graft rejection is a process in which the recipient's immune system attacks the transplanted organ or tissue due to genetic and molecular incompatibility (Medawar, 1948; Billingham & Medawar, 1951; Patel & Terasaki, 1969). Rejection and its course is a result of complex interaction of many different factors, producing a unique pattern of conditions and circumstances for every individual allograft recipient. Nevertheless, the principal mechanisms leading to rejection development, as well as the immune components involved in this process, are defined and described.

Pathways of allograft antigen recognition

There are three basic mechanisms of antigen allorecognition leading to activation of the recipients´ T and B cells and resulting in graft rejection.

The direct pathway

The direct pathway of allorecognition is based on the capability of the host CD4+ and CD8+ lymphocytes to recognize a combination of donor alloantigen molecules and donor MHC class I and class II molecules present on the Antigen Presenting Cells (APC) of the organ donor. After transplantation, the donor's APC, which are stimulated by pro-inflammatory cytokines in response to ischemic and reperfusion injury of the graft, stimulate the host immune response by presenting donors' endogenous peptides and antigens loaded into intact MHC of donor APC to the host T cells. Interestingly, while in normal immune response to any infection less than 0.1% of host T lymphocytes are specific for a given self MHC and foreign peptide complex, in the alloimmune response, a much higher number of T cells (around 10%) recognize allogeneic MHC-peptide complex, which leads to a strong alloimmune reaction. This phenomenon could be at least partially explained by cross-reactivity of T cells specific to viral antigens and alloantigens (D'Orsogna et al, 2012).

Direct allostimulation is associated with a predominance of Th1 activation and is sensitive to increased immunosuppression. The direct pathway of allorecognition is evidently the basic

mechanism underlying early acute cellular rejection. The following evidence supports this contention: 1. the high precursor frequency of T cells capable of interaction with allogeneic MHC; 2. enhancement of ACR by pretreatment of donors with agents that increase the number of donor APCs; 3. ability of T-cell lines specific for direct recognition of allogeneic MHC molecules to induce ACR in immunocompromised hosts; 4. depletion of the donors APC from the graft prior to transplantation resulting in graft tolerance or at least reduced acute rejection, while development of chronic rejection is neither delayed nor suppressed (Jiang et al., 2004).

The indirect pathway

In the indirect pathway of allorecognition, host APC, which infiltrate the graft as part of the post transplant inflammation, recognize donor MHC as a foreign antigen, process it and present peptides on their own MHC molecules. Following migration to the lymphoid organs, these MHC-peptide complexes are presented to host T cells. This pathway is similar to the process by which a foreign infectious antigen is presented to T lymphocytes and involves a lower number of T cells than the direct response (Ingulli, 2010). The indirect alloresponse is oligoclonal and initially involves only a few dominant antigen peptides on donor MHC class II determinants. However, the indirect presentation can be associated with "epitope spreading" of new determinants on donor MHC and tissue-specific antigens or "autoantigens". The indirect pathway is dominant for the late post-operative allosensitization and chronic rejection (ChR) development (Gökmen et al., 2008).

The semi-direct pathway

Recently, the third, so-called the semi-direct pathway of allorecognition has been described. It is based on the host APC capability to acquire a significant amount of intact MHC class I and II molecules from donor APC and from endothelial cells of the graft through cell-to-cell contact or from exosomes secreted by other APCs. Recipient APCs then become chimeric for MHC and are able to prime both CD4+ and CD8+ donor T cells (Jiang et al., 2004; Smyth et al., 2006).

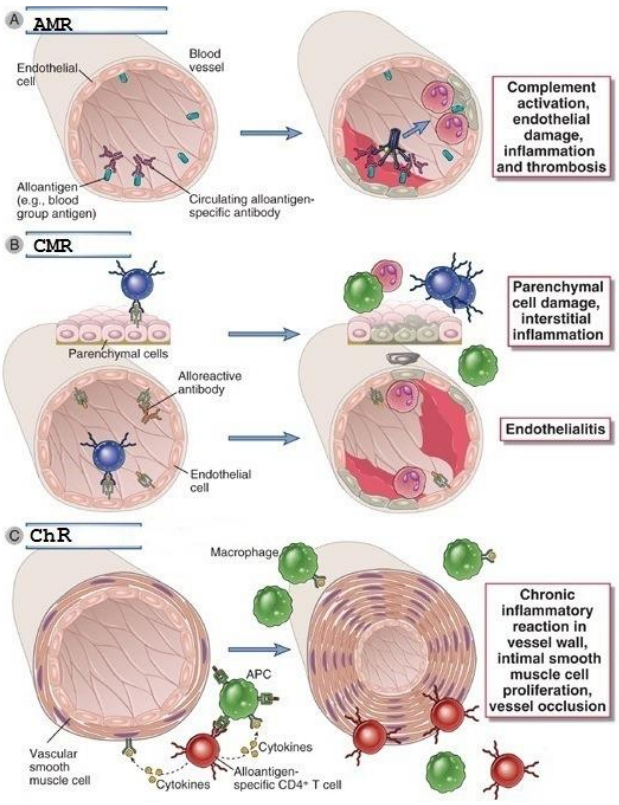

Figure 1. Mechanisms of rejection. A. AMR – Antibody-mediated rejection; B. CMR – Cells-mediated rejection; C. ChR – Chronic rejection (adopted with changes from Abbas et al., 2012)

Acute cellular rejection

Allosensitized T-cells mediate acute cellular rejection (ACR) by responding to alloantigens, predominantly foreign MHC molecules presented on vascular endothelial and parenchymal cells of the graft. T-cells directly stimulated by donor APCs in recipients´ lymphoid organs or in the graft, start to produce IL-2, IFN-gamma, TNF-alpha, IL-15 which initiate lymphoid cells proliferation and migration into the graft (Figure 1, B). Thus, at histologic examination, acute cellular rejection is characterized by interstitial infiltrations of mononuclear cells which invade and destroy graft components (Costanzo et al., 2010). These cellular infiltrates are markedly enriched for CD8+ CTLs specific for graft alloantigens. Experimentally, alloreactive CD8+ CTLs can be used to adoptively transfer acute cellular graft rejection. As in vascularized grafts such as kidney grafts, endothelial cells are major targets of acute rejection and microvascular endothelialitis is a frequent early finding in grafts undergoing acute rejection episodes which, if left untreated, will likely result in acute graft failure (Abbas et al., 2012).

Antibody-mediated acute and chronic rejection

Acute antibody-mediated (also known as humoral) rejection (AMR) is a complication which occurs due to the presence of donor-specific antibodies (DSA) in the recipient blood stream. The first described type of such rejection was the so called “hyperacute” rejection which is no longer accepted by the Banff classification for the histological diagnosis of rejection. Hyperacute rejection is initiated within minutes to hours after transplantation and is mediated by preexisting highly reactive DSA which interact with alloantigens on the donor endothelial cells causing complement activation and graft endothelial cell injury. This leads to exposure of subendothelial basement membrane proteins and platelet activation. This cascade results in intravascular thrombosis and fast irreversible ischemic graft damage. For decades, a pre-transplant complement-dependent cytotoxicity (CDC) cross-matching test has been widely used at clinics to avoid hyperacute rejection caused by these antibodies (Patel and Terasaki 1969; Abbas et al., 2012).

In some cases, low antibody levels or antibodies specific to donor endothelial antigens undetectable by CDC may pre-exist in the recipient or de-novo DSA production by memory alloreactive B-cells may occur shortly after transplantation. These antibodies may cause acute antibody-mediated rejection within the first months after transplantation.

There are four categories of symptoms of AMR (Colvin & Smith, 2005; Mengel et al., 2012):

1. Functional: graft dysfunction; total graft failure
2. Morphologic: acute tubular injury, neutrophils and/or mononuclear infiltrates in peritubular capillaries and/or glomeruli, and/or capillary thrombosis; intimal arteritis/fibrinoid necrosis/intramural or transmural inflammation in arteries
3. Immunohistologic: C4d deposits in graft tissue; Ig and/or complement in arterial fibrinoid necrosis
4. Serologic: circulating antibodies to donor HLA or other donor-specific or cross-reactive antibodies at the time of biopsy

In kidney transplantation, besides graft dysfunction, at least one sign from each group should be present for AMR diagnosis. In heart, lung and liver transplantation, the criteria may vary between centers, but in most cases a combination of graft dysfunction, C4d deposits and DSA presence is necessary for the diagnosis of acute AMR.

Until recently, it had been accepted that complement activation by HLA-specific antibodies is the principal mechanism of AMR. C4d is a degradation product of the classic complement pathway which may bind to the endothelial and collagen basement membranes. However, while

C4d remains a marker of an antibody-antigen binding and complement activation, recent evidence indicates that, in a considerable number of cases where DSA presence is combined with signs of antibody-mediated injury in biopsies, C4d deposits are undetectable. This alternative rejection phenotype, so-called C4d negative antibody-mediated rejection, is characterized by high intragraft endothelial gene expression, alloantibodies, histologic signs of acute (or chronic) AMR and poor graft outcomes. C4d-negative AMR is reported to be twice as common as C4d-positive AMR and may often be diagnosed where non-HLA antibodies are present (Sis & Halloran, 2010; Haas, 2012). The recent Banff Working Group Meeting report highlights the necessity of defining criteria for early diagnosis of C4d negative rejection (Mengel et al., 2012).

While the risk of acute rejection occurrence in patients with sufficient immunosuppressive therapy and uncomplicated post-transplant course decreases over time, the risk of chronic rejection remains high and even grows (Lentine et al., 2012).

The main immunological characteristics of the ChR are prevalence of CD4+ TH2 cells in biopsies, influx of activated recipient macrophages, presence of DSA and non-DSA and comparatively poor response to immunosuppression. All these parameters indicate that, in chronic allograft rejection, humoral mechanisms become the key factor of irreversible graft injury (Weiss et al., 2008; Loupy et al., 2012). Besides vascular damage caused by acute cellular and antibody-mediated rejection episodes, chronic rejection onset may be provoked by injuries caused by infection, metabolic abnormality and ischemia (Regele et al., 2002). Although the injured epithelium is repaired, the repairing mechanisms are evidently insufficient from a long-term perspective. The progressive damage-repair-damage pathological process gradually remodels arteries and basement membranes due to excessive endothelial proliferation and elevated fibrogenic growth factors production. This results in fixed and irreversible anatomical lesions that permanently compromise graft function (Colvin & Smith, 2005). Histological changes typical for ChR (Figure 1, C), lead to progressive narrowing of arteries, known as "graft vascular disease" or "obliterative arteriopathy" (OA). Obliterative arteriopathy damages the allograft by altering the arterial blood flow, thus predisposing to chronic ischemic damage and infarction followed by interstitial inflammation, destruction of epithelial-lined conduits (such as bronchioles in lungs and bile ducts in the liver), fibrosis and scarring in the graft (Cai & Terasaki, 2005; Nath et al., 2010). In kidney recipients, ChR is described as chronic allograft nephropathy (CAN); in heart transplants, ChR manifests as cardiac allograft vasculopathy (CAV), which usually leads to ischemic episodes; in transplanted lungs, ChR causes bronchiolitis obliterans syndrome (BOS);

while in liver transplants, ChR is characterized by the vanishing bile duct syndrome (Visner & Goldfarb, 2007).

There are a few main problems which make chronic rejection one of the crucial factors negatively influencing the long-term post-transplant prognosis:

1. Multiple mechanisms of the ChR initiation, development and graft damage require complex therapy;
2. Chronic rejection is in most cases a relatively slow process that often develops sub-clinically, which makes detection of ChR onset quite complicated;
3. Graft damage and function loss caused by ChR, unlike acute rejection, is irreversible;
4. The risk of ChR increases with time after transplantation;
5. Effective protocols for ChR treatment or prevention are still lacking.

Due to these factors, chronic rejection (C4d positive or negative) is the most common cause of late kidney graft dysfunction or loss (Sis & Halloran, 2010).

Genetic factors influencing the outcome of organ transplantation

The HLA complex

General characteristics

The antigens responsible for rejection of genetically disparate tissues are termed histocompatibility antigens. Histocompatibility antigens are encoded by numerous loci (genes) located in the Major Histocompatibility Complex (MHC) and are responsible for the most vigorous allograft rejection reactions. In humans, the MHC is called the Human Leukocyte Antigen (HLA) system. The genes of the HLA system are sited on the short arm of chromosome 6 and are grouped into classes I, II and III. The HLA class I and class II genes are highly polymorphic with more than 7000 variants (alleles) (according to IMGT/HLA Database, 2011). The genes encoding the heavy chains of the class I histocompatibility antigens, HLA-A,B, and Cw, and those encoding the alpha and beta chains of the DR and DQ class II antigens are closely linked and inherited together as haplotypes. The distribution of alleles and specific haplotypes differs among populations and ethnic groups (Bontadini, 2012).

HLA molecules belong to a group of molecules known as the “immunoglobulin superfamily” and control immune response through recognition of ‘‘self’’ and ‘‘non-self’’. The biologic function of MHC molecules is to present antigen peptides to T lymphocytes, which places these molecules among the key players in immune response initiation. The domains of MHC molecules

distal to the cell membrane form a peptide binding groove that restricts peptide binding to ones with appropriate size and sequence motifs. Changes in the DNA sequences of the HLA genes, particularly if located in the exons coding the antigen presentation domains, affect peptide binding and subsequent antigen presentation. These gene variations result in variations of HLA molecules which may be recognized by the immune system as foreign.

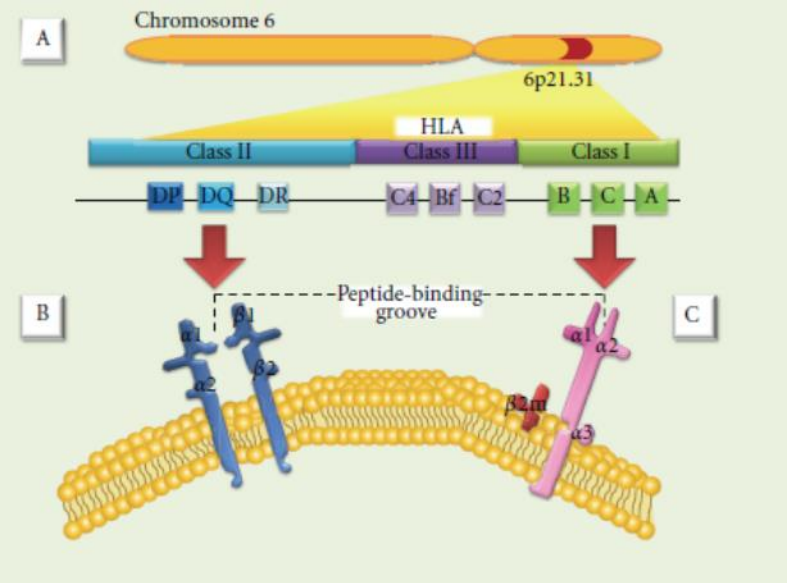

Figure 2. A. MHC (major histocompatibility complex) B. Class II antigens. C. Class I antigens. (adopted from Ayala et al., 2012)

Class I HLA antigens are present on all nucleated cells and are composed of a 45-kd α heavy chain encoded by genes of the HLA-A, HLA-B, or HLA-C loci. The heavy chain is non-covalently associated with a 12-kd non-polymorphic chain - β2 microglobulin encoded on chromosome 15 which structurally supports the heavy chain. HLA molecules are divided into three regions: extracellular, intramembranous and intracellular. The extracellular region has three domains: α1, α2 and α3 (Figure 2, C). The α1 and α2 domains form the antigen-binding cleft, the floor of which is formed by a β-pleated sheet that is overlaid by two α-helical walls. These two α domains are coded by the second and third exon of the HLA class I gene. The high polymorphism of HLA class I antigen is located in these two domains which are responsible for peptide binding and interaction with T cell receptors. HLA class I molecules are recognized by CD8+ T lymphocytes, presenting them endogenous peptides derived mainly from virus proteins and defective intracellular proteins. Several HLA class I molecules (HLA-Cw, HLA-Bw4 and HLA-A3/A11) are also known to be ligands for NK-cells activating and inhibiting Killer Immunoglobuline-like Receptors (KIR). The expression levels of corresponding HLA class I

molecules regulate NK cells reactivity by the "missing-self" principle (see the Section "The KIR complex").

Additional (non-classical) class I molecules, encoded by the "non-classical" HLA-E, -F, -G, -H loci, show limited variability and tissue distribution. They are involved in anti-cancer immune surveillance (HLA-E), peptide fragments presentation to γδ T cells and immunological tolerance of a mother to her fetus (HLA-G) (Ayala et al., 2012).

MHC class II antigens are expressed on antigen-presenting cells, i.e. B lymphocytes, activated T lymphocytes, monocytes, macrophages, Langerhans cells, dendritic cells, and under certain conditions on endothelial and epithelial cells. HLA Class II molecules are heterodimers composed of non-covalently associated α and β polypeptide chains. Each of them has two domains. Highly conserved α2 and β2 domains interact with the CD4 molecule of the T lymphocyte, while the highly polymorphic α1 and β1 domains form the peptide binding region (Figure 2, B). There are 3 major HLA class II proteins - HLA-DP, HLA-DQ, and HLA-DR. The α and β chains of MHC class II molecules are encoded by loci A and B, which are closely linked as pairs of genes (i.e., DRA/DRB; DQA/DQB; and DPA/DPB). The HLA-DP and –DQ loci contain polymorphisms in both the A and B genes coding the α- and β-chains , whereas in the HLA-DR molecule, only the DR β chain (DRB gene) is polymorphic, while DR α chain (DRA gene) has very low polymorphism (McCluskey et al., 2003). MHC haplotypes vary in their overall structure and in their gene content. There are nine DRB loci consecutively numbered from DRB1 to DRB9, but the number of DRB loci present on any one haplotype varies. HLA class II molecules are recognized by CD4+ T lymphocytes and their main role is to present antigens derived from extracellular proteins (Ayala et al., 2012).

Due to the involvement in basic reactions of adoptive immunity, the HLA complex is a powerful co-player in a wide range of autoimmune and infectious diseases and immunological dysfunctions.

The HLA complex in transplantation

The degree of immune disparity between the recipient and donor may influence the incidence of rejection and allograft survival. In all three pathways of allorecognition, the donor's HLA antigens alone or in combination with a bound peptide act as strong antigens, activating the recipients' CD8+ and CD4+ T-cells and B lymphocytes, thus playing a key role in acute cellular and antibody mediated rejection.

Once sensitized against particular incompatible HLA antigens, the recipient immune system remains reactive against these antigens even in the case if the antigen exposure is ceased, because of the generation of memory T and B cells. HLA allosensitization occurs not only as a result of transplantation, but also after blood transfusions and pregnancy (in these cases, the mother may become reactive against the father's HLA antigens).

HLA matching is an important factor in most European kidney allocation algorithms used up to the present. It is generally accepted that a HLA-A, -B, and -DR fully matched organ is the best choice for kidney transplantation. Nevertheless, due to increasing demand for organs and insufficient number of available organ donors, the number of partly mismatched transplantations and a median number of HLA mismatches within donor-recipient pairs are growing. Ceteris paribus, both graft and patient survival after kidney transplantation from diseased donors are significantly better in patients with HLA-compatible grafts, compared to patients' transplanted with incompatibilities (Opelz & Döhler, 2007; Johnson et al., 2010). In transplantation from living donors, it is still a matter of debate to what extent HLA mismatching influences graft outcomes. Due to a pronounced favorable effect of the short ischemia time in kidney transplantation from a living-donor, the role of HLA mismatching in early complications, such as delayed graft function and acute rejection, is considered negligible and in most cases it is not taken into consideration. However, recent evidence suggests that HLA mismatches may significantly decrease long-term survival of allografts taken from living-donors (Cecka, 2008; Rizzari et al., 2012).

HLA-DR incompatibility between donor and recipient is the most unfavorable, especially within the first post-transplant year, while during subsequent years, the impact of all three loci on a graft survival was found to be equivalent and additive (Opelz and Döhler, 2007). Doxiadis and colleagues suggest that the effect of HLA-A and HLA-B matching is more pronounced in patients with complete HLA-DR match, whereas incompatibilities in HLA-DR locus minimize additive positive impact of HLA-A and B matching. This was observed in both first transplant and re-transplant patients (Doxiadis et al., 2007; Doxiadis et al., 2010).

At present, in kidney transplantation HLA matching in all three loci, HLA-A, B and DR, remains desirable and in case it cannot be achieved, HLA-DR matching is the first choice in most of kidney allocation algorithms.

In transplantation of solid organs other than kidneys, the HLA matching effect is less studied and pre-transplant HLA matching is less regarded because of the significantly shorter permissible ischemia time and is insufficient for pre-transplant donor HLA typing. Nevertheless, retrospective

studies revealed a highly significant impact of HLA compatibility on the graft outcome in heart transplant recipients. Kaczmarek and colleagues reported that HLA-DR matching improves 3-year heart allograft survival by 25% (Kaczmarek et al., 2006), while data from the ISHLT registry indicate that, in addition to HLA-DR, mismatches at the HLA-B locus reduced 10-year heart allograft survival (Taylor et al., 2006; Weiss et al., 2008).

The KIR complex

General characteristics

Killer immunoglobulin-like receptors (KIRs) are members of the group of cell-surface molecules that activate or inhibit natural killer cells (NK) and subsets of cytotoxic T cells through interaction with ligands on the surface of target cells. The KIR locus, containing a family of polymorphic and highly homologous genes, maps to chromosome 19q13.4 within the 1 Mb leukocyte receptor complex (LRC). KIR genes are tandemly arrayed over about 150 kb and gene content varies between haplotypes (Boyton et al., 2007; Campbell et al., 2011).

The KIR nomenclature accounts for the proteins' structure: each KIR molecule consists of two or three extracellular immunoglobuline domains (2D and 3D molecules respectively), a transmembrane part and a short (S) or a long (L) intracellular tail. KIRs with long cytoplasmic tails are inhibitory by virtue of the tyrosine-based inhibitory motifs (ITIMs) connected with them. Short-tailed KIRs transmit activating signals through the interaction with the DAP-12 adaptor molecule. The only exception to this short/long-tailed rule is KIR2DL4, which is a unique activating KIR. The structure of KIR genes basically corresponds to the molecule structure: the signal sequence is encoded by exons 1 and 2. Each Ig domain (D0, D1, and D2) corresponds to a single exon – 3, 4 and 5 respectively, the linker and transmembrane fragment of each KIR molecule is encoded by exons 6 and 7, respectively, and the cytoplasmic domain is encoded by exons 8 and 9 (Campbell et al., 2011).

To date, 17 distinct KIR gene loci are known including the frame genes (KIR2DL4, 3DL2 and 3DL3) that are present in virtually all haplotypes and two pseudogens (2DP1 and 3DP1) which do not encode cytoplasmic tails. In general, the KIR genes can be arranged into two haplotypes, A and B. Group A haplotypes have only one activating receptor, which is in most cases KIR2DS4, have low variation in gene content but genes have extensive allelic variability. In contrast, group B haplotypes contain two to five activating receptors and have a substantial gene content variability; however the genes are less polymorphic than group A genes. Moreover, individual NK cell clones

from any given individual can show different patterns of KIR receptor expression which also contributes to a remarkable variety of individual KIR repertoires (Campbell et al., 2011). Apart from individual variability, representation of some particular KIR genes varies between populations (Middleton & Gonzelez, 2010).

Ligands for KIR include human leucocyte antigen (HLA)-C, HLA-A and B antigens and, in the case of 2DL4, HLA-G. KIR molecules with three Ig-like domains (KIR3D) are mostly involved in recognition of HLA-A and -B alleles; whereas receptors with two Ig-like domains (KIR2D) bind HLA-C molecules (Table 2). The alleles of the HLA-C locus can be divided into two groups of ligands (C1 and C2) by the amino acid present at position 80 of the molecule with approximately 50% of alleles being in each group. HLA-C2 is more inhibiting to NK cell function than HLA-C1. For some KIRs, the corresponding ligands are still unknown (Boyton et al., 2007; Middleton & Gonzelez 2010).

Receptors	Ligands	Group
2DL2, 2DL3, 2DS2, 2DS3	HLA-Cw*01/03/07/08/12/13/14/1507/1601	C1 (Ser77, Asn80)
2DL1 2DS1	HLA-Cw*02/04/05/06/1242/15/1602/17	C2 (Asn77, Lys80)
KIR2DS4	HLA-C *0501/*1601/0202; A*1102	C1, C2
3DL1	HLA-Bw4	-
3DL2	HLA-A3/11	-
2DL4	HLA-G	

Table 1. KIRs and their known ligands

Healthy cells are protected from spontaneous NK-cell-mediated cytolysis by interaction of HLA class I molecules and inhibitory KIRs, while other KIRs stimulation leads to activation of NK cells. Every NK cell expresses at least one inhibitory receptor to a self-HLA class I molecule. The balance between inhibitory and stimulatory signals sets the threshold of NK cell functional competence. Downregulation of HLA class I expression on the target cells due to viral infections, neoplastic transformations or stress, reduces inhibitory signaling thus permitting NK cells to

eliminate these target cells. This phenomenon is known as the 'missing-self' hypothesis (Gardiner, 2008; Rajalingam & Gebel, 2011).

Thus, KIR play an important role in the control of the immune response which would explain the associations observed between certain KIR genes and autoimmune and infectious diseases like rheumatoid arthritis (Yen et al., 2001), control of HIV and some other diseases progression (Boyton et al., 2007). It has also been reported that KIR/HLA repertoire may significantly influence the results of bone marrow and umbilical cord stem cells transplantation and leukemia relapse (Velardi, 2008; Willemze, 2010).

The KIR complex in organ transplantation

NK cells use a highly specific and complex target cell recognition receptor system consisting of inhibitory and activating receptors. The secretion of IFN- γ and TNF-α by NK cells is likely to induce and upregulate the expression of MHC molecules and costimulatory receptors on APCs, thus promoting the maturation of professional APCs (dendritic cells and B cells) and presumably nonprofessional APCs (endothelial cells). Analysis of activating and inhibitory receptors and their ligands in donor-recipient pairs revealed that anti-donor NK-cytotoxicity is present in patients with at least one activating KIR that recognizes donor HLA, while recipients without any activating KIR that could bind a donor HLA did not show any similar trend (Vampa et al., 2003). It has also been shown that kidney allografts homozygous for HLA-C group 2 alleles may have a better outcome than grafts which are HLA-C heterozygous or homozygous for HLA-C group 1. Moreover, certain KIR/HLA class I ligand combinations between donor and recipient demonstrated significant influence on graft short and long-term outcome after renal transplantation even in HLA matched pairs (Kunert et al., 2007; van Bergen et al., 2011).

In liver transplantation, KIR donor-recipient mismatch has been described as a risk factor for acute graft rejection (Bishara et al., 2001). Acute liver allograft rejection incidence is also influenced by KIR-ligand heterozygosity. For example, presence of at least one HLA-C group 2 allele in the donor is associated with a significantly improved long term graft and patient survival. HLA-C group 2 homozygous liver allografts demonstrate 26.5% better survival at 10 years compared with transplants homozygous for HLA-C group 1 alleles (Hanvesakul et al., 2008). HLA- C1/C2 group heterozygous donors are best accepted in C1/C1 patients than in C2/C2 recipients who experience a high rate of acute rejection (Bishara et al., 2005; López-Alvarez et al., 2009).

No association has been found so far between KIR repertoire and acute lung transplant rejection, whereas KIR haplotype A has been described as a risk factor for BOS development (Kwakkel-van et al., 2008).

Interestingly, KIR repertoire may influence the post-transplant course in other ways besides direct participation in the rejection process. In the post-transplant period, the number of activating KIR genes in recipient's genotype may inversely correlate with CMV infection and thus kidney recipients with B haplotypes may be at a lower risk of CMV infection (Stern at al., 2008).

The extremely high variety of KIR and HLA haplotypes, as well as the fact that the strength of signals triggered by KIR-HLA interactions depends on the sequence polymorphism of KIR receptors, HLA ligands and HLA-loaded peptides, complicates the design and analysis of studies focusing on the role of KIR genes in solid organ transplantation. These studies also meet such challenges as small patients groups with particular KIR-HLA combination and poor KIR-HLA matching in retrospectively studied cohorts.

Basic techniques for HLA and KIR genotyping

PCR-SSP

For sequence-specific probe (SSP) typing, unique PCR oligonucleotide primer pairs are used to selectively amplify each allele at each locus. Only the oligonucleotide primer pair with an identical sequence to alleles (or allele groups) carried by the individual is amplified. The presence of specific amplicons is then detected by gel electrophoresis. Comparison with molecular size ladders confirms that appropriately-sized amplicons were obtained and internal negative control excludes reagent contamination with irrelevant DNA. Depending on the primers used for testing, the method may be used for low, medium or high resolution typing.

PCR-SSOP

For sequence-specific oligonucleotide probe (SSOP) typing, a single PCR reaction with primers specific for the conserved region of the particular locus is used to amplify that particular locus. The amplicon is then detected with oligonucleotides, with length mostly about 20 bases, which is optimal for complementary hybridization. Hybridization with oligonucleotides that are anchored on a solid phase, such as support membrane or microbeads, is followed by a detection step which may be either enzyme-linked assay or immunofluorescent labeling. This method may be used

mostly for low and medium resolution, although high resolution typing with Luminex-based SSO technology has become recently available.

Sequencing

This technique directly determines the nucleotide sequences of the allele, thus allowing exact allele assignment. DNA sequencing is performed by using 2',3' dideoxynucleotides as substrates. When such nucleotide is incorporated at the 3' end of the growing chain, chain elongation is stopped selectively at A, C, G, T. Four different dyes are used to identify the A, C, G, and T dideoxynucleotides and each dye emits light at a different wavelength when excited by a laser. Because incorporation of the labeled dideoxynucleotides is random, each of them stops the elongation at different spots, where complementary nucleotide is present and subsequently many DNA fragments of differing sizes are generated. These DNA fragments are separated by capillary electrophoresis and all four bases at the fragment ends can be detected and distinguished through the specific fluorescence emitted. Sequence analysis software analyses the nucleotide sequence and compares it with a database including the sequences of all known alleles.

In our work, we used mostly PCR-SSP and PSR-SSOP techniques for HLA and KIR genotyping.

Molecular factors influencing the outcome of organ transplantation

HLA-specific antibodies

General characteristics

At the beginning of the immunogenetics era in transplantation, rejection of ABO compatible allografts was attributed exclusively to cellular reactions. In 1969, however, Patel and Terasaki published a landmark study where they unequivocally demonstrated that the presence of pre-formed recipient antibodies to antigens expressed on donor leukocytes are a major risk factor for hyperacute rejection and graft loss (Patel & Terasaki, 1969).

HLA-specific antibodies develop in 25% of renal transplant recipients and are directed against antigen epitopes of class I and/or class II HLA molecules. Their production in most cases is a result of allosensitization, i.e. contact of the immune system with foreign HLA antigens, which is usually a result of blood transfusion, pregnancy or transplantation. However, surprising reports

recently published claim that anti-HLA antibodies may be found even in healthy males with no conventional sensitizing events in the past. In most cases, they are reactive against HLA antigens that are rare in the general population. These so-called "natural" antibodies are supposed to be directed against cross-reactive epitopes found in microorganisms, ingested proteins and some allergens (El-Awar et al., 2009).

With regards to the donors´ HLA phenotype, HLA-antibodies are divided to donor-specific antibodies (DSA) and non-donor-specific antibodies (NDSA). Antibody-mediated rejection is attributed mostly to DSA, although NDSA may also participate in acute AMR and chronic rejection due to cross-reactivity against shared epitopes of different HLA molecules (Cai et al., 2006; Briggs et al., 2009).

Based on the idea of cross-reactivity, the concept of CREGs – Cross-Reactivity Groups – has been proposed. According to this concept, HLA antigens are divided into groups based on the particular immunogenic epitopes they share. This concept is focused on HLA-specific antibody reactivities and cross-reactions rather than on conventional allelic HLA matching. As CREGs, unlike particular HLA alleles, are present equally in all ethnic groups, this concept allows for finding more "well-matched" organs for recipients from different populations and bearing rare HLA haplotypes.

The conventional antibody testing is the panel-reactive antibodies (PRA) test which is still used for evaluation of the recipients' HLA antibody profile before including the patient into the transplant waiting list. PRA testing is performed as complement-dependent cytotoxicity reaction (CDC) (see the Section "Methods for molecular factors detection and evaluation") of patients' serum with a panel of cells obtained from healthy individuals. This panel is usually composed of 50-100 cells selected with respect to HLA allele frequencies of the local population. The reaction pattern shows the specificity of the antibodies present in the serum and the results are expressed as a panel reactive antibody (PRA) value defined as the percentage of cells in the panel to which the serum reacts.

Not long ago, new highly sensitive so-called solid-phase assays have been introduced, improving antibody detection (see the chapter "Methods of molecular factors detection and evaluation"). The development and implementation of these assays utilizing purified HLA antigens allow for discrimination of HLA antibodies according to their particular antigene specificity and antibody detection in concentrations undetectable by CDC (Lee &Ozawa, 2007; Amico et al., 2008). Solid-phase assays are also capable of detecting non-complement binding

antibodies, which is important, because recent studies indicate that total pre-transplant HLA-targeting DSA is a better marker of the increased risk of rejection and graft failure than the fraction of only complement-binding DSA (Wahrmann et al., 2009; Hönger et al., 2010). Despite these advantages, the extreme sensitivity of solid phase assays have raised discussion of whether all antibodies detected by these methods in patient serum are equally important in rejection risk evaluation or if low antibody concentrations and certain antibody specificities (such as mentioned above "natural" antibodies specific to rare HLA antigens) might be accepted as clinically irrelevant.

Although in most cases HLA-specific antibodies of the IgG isotype are responsible for the development of AMR and are taken into consideration in pre-transplant examination, Stastny et al. recently reported that donor-specific IgM antibodies may also increase the risk of AMR in both kidney and heart transplant recipients (Stastny et al., 2009).

HLA-specific antibodies before transplantation

As indicated above, the first pre-transplant antibody CDC testing was implemented to avoid hyperacute rejection in patients pre-sensitized against donor HLA antigens. Later, it was found that the presence of pre-transplant antibodies negatively influences graft survival and, in some cases, the incidence of early acute rejection may be caused by alloantibodies (Cai & Terasaki, 2005). For that reason, pre-transplant positive CDC cross-match is in general a contraindication for kidney transplantation.

It has been demonstrated that antibodies detected by solid-phase assays may be predictable for acute rejection episodes, and also, especially if analyzed together with early AMR incidence, may be very beneficial in long-term graft survival prediction (Amico et al., 2009; Lefaucheur et al., 2010).

The simultaneous presence of both HLA class I and class II before transplantation significantly increases AMR in HLA mismatched grafts (3 or more A+B+DR mismatches) (Süsal & Opelz, 2002; Süsal et al., 2009) and worsens 10-year allograft survival (Otten et al., 2012), although it is still a matter of discussion whether both class I and class II pre-transplant HLA antibodies are equally important. For example, in a number of studies, pre-transplant HLA class I alone were shown to be correlated with delayed graft function and higher acute AMR incidence (Süsal et al., 2009; Riethmüller et al., 2010).

Interestingly, antibodies not only to HLA A, B, and DR antigens may be involved in kidney allograft rejection, but also pre-existing DSA against DP antigens are able to cause acute graft injury and failure, which raises the question of whether conventional A, B and DR matching is enough to provide the best compatibility between donor and recipient (Billen et al., 2010; Gilbert et al. 2011).

Accumulating evidence suggests that pre-formed HLA antibody might be co-players not only in AMR but also in CR episodes in kidney transplantation (Cinti et al., 2009; Huh et al., 2012).At present, the association between pre-transplant HLA class I antibodies and CR has been reported while the role of HLA class II antibodies remains unclear (Cinti et al., 2009).

In heart transplant recipients, pre-transplant DSA to both HLA class I and class II are associated with early development and higher incidence of acute rejection episodes as well as with impaired allograft survival (Stastny et al., 2007; Chin, 2012). For some time, the effect of pre-transplant HLA antibodies on liver allograft was considered negligible. Nevertheless, recent studies demonstrated that pre-formed class II or a combination of class I and II antibodies as detected by Luminex are associated with allograft rejection and decreased first year survival in first transplant recipients (Castillo-Rama et al., 2008). In re-transplant liver recipients, pre-existing class I specific antibodies are associated with increased graft-loss while class II antibodies may be associated with a higher incidence of chronic rejection (Goh et al., 2011; O'Leary et al., 2011).

All in all, even though most studies show a relation between pre-transplant HLA DSA and impaired graft outcomes, the presence of HLA antibodies in pre-transplant sera may not inevitably result in graft loss. Further studies are needed to clarify the clinical significance of different pre-transplant antibodies according to their specificity, class, concentration and applied method of antibody detection.

HLA-specific antibodies after transplantation

A number of studies support the idea that de novo DSA production is associated with acute graft rejection (Scornik et al., 2007; Cooper et al., 2011). It has also been suggested that the post-transplant dynamics in concentration and specificity of pre-formed DSA might be informative in AMR risk assessment. For instance, patients with high DSA before and after transplantation are at significantly higher risk of rejection than those who had high pre-transplant and low post-transplant DSA (Burns et al., 2008).The same trend has been observed in patients who underwent desensitization before transplantation: patients with significant decrease of DSA post-transplant

were at a lower risk of rejection than those whose DSA remained at a high level (Reinsmoen et al., 2008). Changes in DSA levels are also helpful about the course of an AMR episode. Patients with significant reduction of DSA after diagnosis and following immunosuppression adjustment have significantly better allograft survival than those whose DSA remain at a high level despite changes in immunosuppressive regimen (Everly et al., 2009).

Post-transplant de novo DSA production varies in a range from 1.6 to 60% of kidney recipients according to different centers and methods used for the post-transplant DSA detection (Akalin & Pascual, 2006). In most cases, post-transplant antibodies specificity is wider than against the particular mismatched HLA antigen(s) which is allegedly due to cross-reacting epitopes of other HLA molecules (Stastny et al., 2009). In some cases, de novo DSA may not be detected in patients' sera but may be eluted from kidney allografts undergoing rejection. Recent data also confirm a significant increase in DSA levels within the first weeks after transplantectomy (up to 80-90% of first transplanted patients become DSA positive) (Doxiadis & Claas, 2006; Billen et al, 2009). Presumably this phenomenon may be caused, on one hand, by elution of antibodies from the graft before its' removal and, on the other hand, by ceasing of immunosuppression (Doxiadis & Claas, 2006; Stastny et al., 2009).

Interestingly, de novo antibodies directed against HLA antigens which are not included into donor-recipient compatibility algorithms, namely HLA-DQ antigen, may also have deleterious effects on renal graft outcome as well as cardiac allograft outcome (Reed et al., 2006).

In heart transplantation, de novo DSA production is associated with impaired graft survival and development of AMR which is often resistant to conventional immunosuppressive protocols (Hodges et al., 2012).

In lung transplant recipients, post-transplant DSA are also associated with recurrent acute rejection and higher grade of rejection episodes (Girnita et al., 2004; Visner & Goldfarb, 2007).

In liver transplantation, the relevance of post-transplant antibodies is still questioned, although recent reports suggest that circulating DSA are associated with AMR and higher incidence of CR (Musat et al., 2011) and withdrawal from the immunosuppressive treatment is successful only in liver recipients free of DSA production (Girnita et al., 2006; Girnita et al., 2010).

HLA-specific antibodies and chronic rejection

Over recent decades, the mechanisms of chronic rejection have been widely discussed with particular attention to the role of HLA-specific antibody persisting in recipient's circulation. An impressive number of studies have appeared recently revealing a linkage between HLA antibody production and chronic rejection in renal, heart, lung and liver transplants (Cai & Terasaki, 2005; Stastny et al., 2007; Visner & Goldfarb, 2007; Lachmann et al; 2009; Lee et al., 2009; Nath et al., 2010; Smith et al., 2011; Iacob et al., 2012). Sometimes it may take years between the occurrence of DSA and graft loss, which is presumably due to a progressive damage-repair-damage effect.

Interestingly, not only DSA are associated with graft failure and ChR incidence but also HLA NDSA presence increases the risk of such events in comparison with antibody-free patients which may be due to cross-reactivity (Lachmann et al., 2009). The possible mechanism of graft-damage by non-cross-reacting NDSA remains unclear.

The allograft endothelium injury caused by DSA and/or cellular rejection episodes may lead to presentation of hidden self-antigens to the recipients' immune system which results in autoimmune response. The range of these self-antigens is wide: myosin and vimentin in cardiac, K-alpha-1-tubulin and collagen-V in lung and angiotensin II type 1 receptor, collagen-IV and VI in kidney transplants. Even transient appearance of anti-HLA antibodies often preceded the development of antibodies to self-antigens (Tiriveedhi et al., 2010; Nath et al., 2010; Saini et al., 2011).

Importantly, while the relative risk of allograft loss due to CR peaks at approximately 6 to 24 months after transplantation and then decreases with time, the relative risk of de novo DSA production and graft loss due to antibody-mediated ChR continuously increases with time after transplantation (Lachmann et al., 2009).

Non-HLA antibodies

General characteristics

Besides HLA antigens, the recipient's immune system may be sensitized by other donor antigens, such as MICA (MHC class I polypeptide-related sequence A), angiotensin type 1 receptor (AT(1)R), collagen-V, glutathione S-transferase T1, vimentin and further yet unknown endothelial or tissue-specific antigens.

Despite the fact that the vascular endothelium of the organ allograft is the “first line” and the main target for the recipient immune response, conventional pre-transplant tests are not aimed at detection of antibodies directed against MICA and other non-HLA endothelial antigens. Cases of hyperacute allograft rejection caused by anti-endothelial antibodies (AECA) have been described and demonstrate that non-HLA antibodies remain a hidden risk factor which may affect transplant outcome especially in immunologically high-risk patients(Grandtnerová et al., 2008; Sun et al., 2008).

The mechanisms of allograft damage caused by non-HLA antibodies are:

Complement activation. Complement-dependent cytotoxicity of non-HLA AECA has been observed in kidney and heart transplant recipients (Sumitran-Holgersson et al., 2002; Rose & Smith, 2009).

Proinflammatory effect. Non-HLA antibodies may participate in acute rejection indirectly by inducing an endothelial cell phenotype that supports leukocyte extravasation through upregulated expression of adhesion molecules, like E- and P-selectin (CD62E/P), VCAM-1 (CD106), ICAM-1 (CD54), inflammatory cytokines (IL-1β, TNF-α) and chemokines (Sumitran-Holgersson, 2008).

Prothrombotic phenotypes. MICA antibody-containing sera from patients demonstrate the ability to induce a pro-thrombotic phenotype of endothelial cells leading to vascular thrombosis and graft loss (Sumitran-Holgersson et al., 2002).

Apoptosis. AECA are capable to promote apoptosis in endothelial cells, decreasing cell viability by 50–60% in vitro (Le Bas-Bernardet et al., 2003).

Among non-HLA antibodies, MICA-specific antibodies are the best studied. MICA antigens are surface stress-inducible glycoproteins expressed on endothelial cells, dendritic cells, fibroblasts, epithelial cells and several tumors, but not on peripheral-blood lymphocytes. MICA is determined by a genetic locus which has by now approximately 60 alleles and is closely linked to the HLA-B locus. MICA antibodies may be found in patients with no previous transplantations. The rate of sensitization to MICA antigens is approximately 6% in the healthy population and 21% in multiparous women (Liapis & Wang, 2011). The frequency of sensitization against MICA appears to increase after transplantation and antibodies against MICA have been found most frequently in recipients who have rejected an organ allograft (Stastny et al., 2009).

Anti-endothelial antibodies other than against MICA have remained less characterized. The reason is that their target antigens are often unknown and, in addition, no reliable tests for their

detection and discrimination have been available until recently (Slavcev, 2012; Dragun et al., 2012).

AECA and MICA-specific antibodies before transplantation

Over a decade ago, it was suggested that non-HLA antibodies might participate in organ allograft rejection. This suggestion was supported by the fact that, in some cases, ABO compatible patients without pre-transplant anti-HLA antibodies but with preformed AECA experienced hyperacute rejection (Sun et al., 2008; Grandtnerová et al., 2008). Later, C4d positive and C4d negative AMR episodes caused by AECAs were described and a correlation between AECA, AMR and graft failure has been demonstrated in wide cohorts of patients (Han et al., 2009; Breimer et al., 2009)

Importantly, not only IgG but also IgM antiendothelial antibodies were found to effect graft outcome. A retrospective analysis of pre-transplant sera from 616 cardiac transplant patients showed that the presence of IgM complement-fixing non-HLA antibodies was associated with early graft failure (Rose & Smith, 2009).

The introduction of the assays capable to distinguish MICA-specific antibodies from other AECA circulating in recipient's blood allowed focusing on these antibodies and exploring their particular role in post-transplant graft injury. It turned out that approximately 15% - 24% (dependent on a center) of patients listed on the kidney transplant waiting list were sensitized against MICA antigens which is much more frequent than among healthy individuals (Zou et al., 2006; Lemy et al., 2010). The role of preformed MICA-specific antibodies in early acute rejection onset is still unclear, although presence of pre-transplant antibodies against MICA antigens was reported to increased probability of renal transplant failure within the first year especially in a well HLA-matched cohort (0 or 1 HLA-A plus HLA-B plus HLA-DR) (Sumitran-Holgersson et al, 2002.; Zou et al., 2007). Therefore, further studies are needed for better understanding to what extent preformed MICA-specific antibodies participate in allograft rejection.

AECA and MICA-specific antibodies after transplantation

Patients whose pre-transplant AECA remained elevated within early post-transplant period are at a greater risk of AMR and are considerably more likely to experience multiple AMR episodes than those whose AECA levels decreased post-transplant (Nakagawa et al., 2002). Currently it is also

recognized that de novo AECAs appearing after transplantation are a significant risk factor for kidney graft rejection and failure (Costa et al., 2010; Sun et al., 2011; Dragun et al., 2012).

It has also been reported that AMR with present AECAs is frequently resistant to steroid treatment and patients producing AECAs are more likely to experience multiple rejection episodes than those who have only HLA-specific antibodies during first year follow-up (Sun et al., 2008).

Recent studies focused exclusively on post-transplant MICA-specific antibodies revealed an association between presence of post-transplant antibodies against MICA and early kidney graft loss in recipients who received well HLA-matched allograft and were free of HLA specific antibodies (Zou et al., 2006; Stastny et al., 2009). A further study revealed a strong correlation between de novo MICA antibodies production in patients who received a MICA mismatched kidney and AMR incidence within first post-transplant year (Cox et al., 2011). Co-presence of MICA and HLA antibodies may significantly compromise graft survival and is a risky combination for early acute rejection occurrence (Panigrahi et al., 2007).

Acute heart allograft rejection also appeared to be influenced by post-transplant MICA antibody production (Suárez-Alvarez et al., 2007; Kauke et al., 2009; Nath et al., 2010). Suárez-Alvarez and colleagues described a significant prevalence of post-transplant MICA antibodies as detected both by CDC cross-matching with MICA expressing cell-lines and by Luminex Single antigen assay in patients who experienced AMR, but were free from HLA-specific antibodies. Upregulated MICA mRNA production in endomyocardial biopsies was associated with de novo MICA antibody appearance which preceded histological evidence of severe rejection (Suárez-Alvarez et al., 2007).

MICA antibodies and chronic rejection

A series of studies exploring the possible relationship between post-transplant MICA antibody production and kidney allograft failure due to chronic rejection revealed that MICA antibodies are an independent risk factor for impaired graft survival. MICA detection is especially helpful in graft failure prediction in patients who also produce anti-HLA antibodies (Mizutani et al., 2005; Mizutani et al., 2006). Terasaki's group reported a close association of the presence of MICA antibodies shortly after the transplantation and chronic kidney allograft rejection within 4 years after transplantation (Terasaki et al., 2007), while Lemy et al. suggested MICA positivity at year 1 as independent risk factor of chronic rejection in patients after kidney transplantation (Lemy et al., 2010; Lemy et al., 2012).

In heart transplantation, MICA antibodies are recognized as a risk factor of the CAV (Kauke et al., 2009). As well as in kidney transplantation, combined post-transplant production of anti-MICA and anti-HLA antibodies were reported to be the most unfavorable prognostic combination for the heart graft survival and freedom from chronic graft rejection (Nath et al., 2010; Zhang et al., 2011).

Soluble CD30

General characteristics

CD30 is a 120-kDa transmembrane glycoprotein that belongs to the tumor necrosis factor receptor superfamily. Cell surface expression of CD30 appears to be restricted only to activated, but not resting, CD4 + and CD8 + T cells as well as B-cells. In healthy individuals, CD30 is detectable only on a small fraction of T lymphocytes (<2%), particularly on CD4+ cells co-expressing the memory marker CD45R0. CD30 is known to be a positive regulator of apoptosis and also has been shown to limit the proliferative potential of autoreactive CD8 effector T cells and thus protects the body against autoimmunity. Recently, CD30 expression has been detected in an immunomodulatory subpopulation of T cells as well as in a subpopulation of dendritic cells (DCs) known to modulate immune responses and tolerance. In addition, CD30+ T cells were demonstrated to produce high amounts of cytokine IL-10 and thus suppressed Th1 response. Besides Th2 stimulation, CD30 is more generally involved in the balance regulation between Th1 and Th2 type immune response (Pellegrini et al., 2005).

The membrane-bound CD30 molecule can be proteolytically cleaved from the cell membrane by the metalloproteinase TNF-α-converting enzyme, thereby generating a soluble form (sCD30) of about 85 kDa.

Low serum levels of soluble CD30 were found in peripheral blood of healthy humans, whereas increased sCD30 concentrations, which appear to reflect an active involvement of CD30 cells, were detected under some pathophysiological situations such as Hodgkin disease, anaplastic large cell lymphoma (ALCL), adult T-cell leukemia/lymphoma (ATL), systemic lupus erythematosus, rheumatoid arthritis and certain viral infections (Schlaf et al. 2007).

Soluble CD30 in transplantation

The first report suggesting pre-transplant sCD30 as a marker for acute rejection was published in 2002 by Pelzl and co-workers and provided evidence that high pre-transplant sCD30 might be a risk factor for acute kidney graft rejection in both previously sensitized (PRA>5%) and in non-sensitized (PRA≤5%) recipients (Pelzl et al, 2002). Further studies also revealed a relationship between high pre-transplant sCD30 levels and poor allograft survival (Cinti et al., 2005; Sengul et al., 2006) that suggested setting a clinically relevant cut-off between high and low pre-transplant serum levels at 100 U/ml. Although mechanisms connecting graft failure and sCD30 levels are not crystal clear: there have been some reports that elevated pre-transplant sCD30 is associated with antibody-mediated rejection (Rajakariar et al., 2005; Vaidya et al., 2006), while other investigators, including our group, did not confirm a relationship between pre-transplant sCD30 and acute rejection episodes (Slavcev et al, 2005; Dong et al., 2006).

An intriguing finding was that sCD30 concentration may influence kidney graft survival independently of PRA and may even be predictive for post-transplant DSA production (Vaidya et al., 2006). Moreover, both PRA and sCD30 have been demonstrated to have a synergic effect on graft outcome (Rodriguez et al., 2007; Wang et al., 2012). Interestingly, in patients with low sCD30, HLA matching influenced graft survival insignificantly, while among patients with elevated sCD30 well-matched group had a significantly better prognosis than a group with poorly HLA matched kidneys (Süsal et al., 2002; Süsal et al., 2003; Spiridon et al., 2008).

In heart transplantation, the role of sCD30 is still uncertain. While it has been reported that elevated sCD30 concentrations are associated with significantly lower graft survival (Frisaldi et al., 2006), other studies did not reveal correlation between pre-transplant sCD30 and transplant outcome (Spiridon et al., 2006; Ypsilantis et al., 2009).

During the first days after kidney transplantation, a significant decrease of sCD30 concentrations is usually observed (Slavcev et al, 2005; Sengul et al., 2006; Halim et al., 2010). Nevertheless, concentrations of sCD30 in patients with acute rejection remain higher than those in patients free of rejection. A significant delay in postoperative sCD30 decrease has been observed in patients who later developed an AR episode (Wang et al., 2007). A number of studies have demonstrated that sCD30 levels measured post-transplant between day 2 and 14 have the greatest predictive value for acute rejection episodes (Dong et al., 2006; Nafar et al., 2009), while measurement in day 30 after transplantation, when post-operative sCD30 concentrations reach the lowest level, is predicative for graft loss (Süsal et al., 2011). As for other organs, it has been reported that liver

recipients experiencing a significant increment of sCD30 within the first week after the transplantation were at a greater risk of the following acute graft rejection (Kim et al., 2006; Fábrega et al., 2007). The most pronounced difference in postoperative sCD30 levels was observed between non-rejecting patients and recipients with established diagnosis of acute rejection, which could support the idea of using post-transplant sCD30 levels not only for rejection risk evaluation but also directly as a marker of the acute rejection onset (Fábrega et al., 2007).

While the importance of sCD30 in prediction of acute rejection has been extensively discussed, it's role in development of ChR is less investigated. Soluble CD30 appeared to be associated with chronic kidney allograft rejection independently of other classical immunological risk factors and may be down-regulated using Tacrolimus treatment (Weimer et al., 2005). In lung transplant recipients, the rise of sCD30 correlates with occurrence of BOS (Fields et al., 2006; Frisaldi et al., 2006; Golocheikine et al., 2008).

Hepatocyte Growth Factor

General characteristics

Hepatocyte Growth Factor (HGF) is a 91 kDa molecule which is secreted as a single inactive polypeptide. The molecule is then cleaved by serine proteases into a 69-kDa alpha-chain and 34-kDa beta-chain. HGF is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. HGF regulates cell growth, motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor (Funakoshi et al., 2003). This cytokine has been initially described as a factor stimulating liver development and regeneration (Schmidt et al., 1995). Later, its' crucial role in other tissues development and regeneration, such as kidney, lung, muscle, and neuronal tissues had been discovered. Antiapoptotic and cardioprotective activity of HGF and its role in angiogenesis and tumorogenesis has been also described (Funakoshi et al., 2003). Concerning interactions with the immune system, HGF inhibits TH1 cytokines (including IFN-γ) and skews monocyte differentiation toward IL-10 production. HGF-differentiated monocytes induce in vitro development of CD4+CD25+FoxP3+ Treg cells that suppress conventional, non-regulatory T cells in a cell contact-dependent manner (Rutella et al., 2006; Isobe et al., 2006).

Significant changes of HGF levels in serum, bronchoalveolar lavage, cerebrospinal fluid and urine were described in relation to such diseases as acute and chronic hepatitis (Shiota et al., 1995); inflammatory lung diseases (Huang et al., 1999); acute heart infarction (Zhu et al., 2000);

acute and chronic renal failure (Taman et al, 1997), etc. In healthy individuals, HGF levels are low but they still may be altered by various factors such as aging, gender and pregnancy.

HGF is widely involved in tissue regeneration after hypoxic organ damage which is, due to the graft ischemia period, a key problem in deceased organ transplantation. Up to now, the graft tissue protective and rejection-suppressive effect of HGF has been demonstrated in animal transplant models (Franquesa et al., 2005; Oku et al., 2012). Additionally, HGF administration may suppress tacrolimus-induced renal toxicity (Takada et al., 1999). This protective effect of HGF might possibly be applied in patients on tacrolimus immunosuppressive therapy.

HGF in transplantation

Although there are numerous studies focused on HGF as a possible component of postoperative graft immune protection, there are only a few studies aimed at evaluation of endogenous HGF levels as a prognostic factor of acute or chronic graft rejection. Post-transplant studies revealed a substantial increase of HGF levels within the first days after transplantation and further decrease approximately to preoperative levels in patients free of rejection (Yoshimura et al., 2002; Aharinejad et al., 2004; Kwiatkovska et al., 2010). A detailed analysis demonstrated that HGF measurement on day 3 – 5 after transplantation may be helpful in discriminating recipients who are more likely to experience early acute rejection from recipients with an uncomplicated post-transplant course (Mahmoud et al., 2007; Li et al, 2008). It has also been shown that HGF levels may increase significantly 1 – 2 days before the rejection episode, which suggests that change in serial HGF measurement might be used as a marker of an up-coming rejection (Aharinejad et al., 2004). The search for the non-invasive way of HGF measurement and acute rejection prediction in renal transplantation lead to the suggestion that HGF detected in urinary samples might be relevant and could be used for detection of impaired kidney allograft function. Indeed, a significant correlation between urine HGF concentrations, blood creatinine levels and diuresis within the first 6 months post-transplant has been observed, although at present there is only one study on this correlation and this finding requires further investigation. (Kwiatkovska et al., 2010).

Among heart transplant recipients, postoperative HGF levels were higher in patients experiencing acute rejection episode and additionally a trend toward higher levels of HGF in patients with higher grades of biopsy findings have been described (Zwirska-Korczala et al., 2005).

In liver transplantation, HGF mRNA measurement in peripheral blood lymphocytes of liver recipients revealed that elevated HGF production as defined by high expression of HGF mRNA in lymphocytes was more sensitive to predict graft dysfunction within the first days after transplantation than other parameters used for this purpose thus far (Dudek et al., 2007).

As HGF is known to be a tissue protective factor which, on one hand, stimulates tissue regeneration and, on the other hand, inhibits fibrosis in the transplanted organ, one may suppose that HGF could play a significant role in graft protection against symptoms of chronic rejection. Indeed, some studies demonstrate that recombinant HGF application at different time points after transplantation ameliorates chronic rejection free survival (Yamaura et al., 2004; Isobe et al., 2006), but the possible role of endogenic HGF levels remains unexplored and unclear. Interestingly, HGF production might be negatively influenced by immunosuppressive drugs (Yamaguchi et al., 1996). Thus, long-term immunosuppression could possibly restrict the protective function of endogenous HGF.

Molecular factors detection and measurement techniques

Complement-dependent cytotoxic cross-match (CDC-XM)

For over 40 years, the complement-dependent cytotoxicity assay (CDC) has been the standard HLA antibody detection cell-based assay. Patient serum is incubated with cells to allow the formation of immune complexes between antigens present on the cells and specific antibodies present in the serum. Subsequently, a rabbit complement binds to immune complexes, thus inducing lysis of cells. Cells are stained with Eosin / or Trypan blue and then evaluated by phase-contrast microscopy. CDC cross-matching is used to assess the presence of DSA in the recipient's blood. It is also used to evaluate pre-sensitization levels in patients on the transplant waiting list by evaluation of their panel-reactive antibodies (PRA).

Flow cytometric cross-match (FCXM)

Flow cytometric crossmatch is a cell-based assay with a several-fold higher sensitivity than the CDC test. Patient serum is incubated with donor cells and then with labeled secondary antibody, which reacts with DSA bound to antigens on donor cells. The fluorescent intensity correlating with the amount of antibody binding to the cells is measured by a flow cytometer. Anti-CD

antibodies marked with different fluorescent dyes and specific to molecular markers of T and B lymphocytes are added for cell discrimination.

Enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays (ELISA) are solid-phase assays designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In the assay, the antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or a matched set of unlabeled primary and conjugated secondary antibodies (indirect detection).

xMap and Flow PRA technology

These are solid-phase flow-cytometry based assays designed for detecting and quantifying substances such as antibodies, peptides, proteins, etc. Microbeads dyed with a unique combination of fluorescent colors and covered with single antigen molecules are used for this test. Beads are incubated with patient's serum, antigen-specific antibodies fix on the antigen and then they are detected by secondary fluorescent anti-human antibody. Fluorescence is measured either by Luminex machine (xMap technology) or by flow cytometer (FlowPRA). Single antigen beads are discriminated according to unique dyes combination and then the mean fluorescence intensity of secondary antibody is measured.

In our research, we used data obtained by all methods mentioned above.

Aims of the thesis

Rejection, whether acute or chronic, remains one of the most frequent causes of solid organ allograft dysfunction and failure in spite of the significant advance in pre-transplant diagnostics and post-transplant treatment. Rejection occurrence may be influenced by a number of genetic and soluble molecular factors. Some of these factors have been chosen for more detailed analysis in this work. The aims of this dissertation are the following:

1. Analyze KIR gene distribution in the healthy Czech population and thus provide a basis for further comparative studies focusing of KIR and their HLA ligands mismatches in transplantation and other clinical studies.

2. Assess the predictive value of pre- and post-transplant HLA antibodies as detected by FCXM, ELISA and/or Luminex for the incidence of acute antibody-mediated and cellular rejection and allograft survival.

3. Evaluate the concentrations of sCD30 as independent predictive factor of acute antibody-mediated renal allograft rejection.

4. Analyze the clinical significance of MICA antibodies in relation with the occurrence of acute cellular and antibody-mediated rejection of the transplanted heart.

5. Study the potential predictive and diagnostic role of HGF concentrations for heart and kidney transplant rejection and to analyze its effects on rejection incidence and allograft outcome in combination with other molecular factors

List of publications

1. *Pavlova Y*, Kolesar L, Striz I, Jabor A, Slavcev A. Distribution of KIR genes in the Czech population. Int J Immunogenet. 2008 Feb;35(1):57-61. IF = 1.522

2. Hollenbach JA, Meenagh A, Sleator C, Alaez C, Bengoche M, Canossi A, Contreras G, Creary L, Evseeva I, Gorodezky C, Hardie RA, Karlsen TH, Lie B, Luo M, Martinetti M, Navarette C, de Oliveira DC, Ozzella G, Pasi A, *Pavlova E*, Pinto S, Porto LC, Santos P, Slavcev A, Srinak D, Tavoularis S, Tonks S, Trachtenberg E, Vejbaesya S, Middleton D. Report from the killer immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA. Tissue Antigens. 2010 Jul;76(1):9-17. IF = 2.33

3. Slavcev A, Honsova E, Lodererova A, *Pavlova Y*, Sajdlova H, Vitko S, Skibova J, Striz I, Viklicky O. Soluble CD30 in patients with antibody-mediated rejection of the kidney allograft. Transpl Immunol. 2007 Jul;18(1):22-7. IF = 2.296

4. *Pavlova YA*, Malek I, Honsova E, Netuka I, Sochman J, Lodererova A, Kolesar L, Striz I, Skibova J, Slavcev A. Hepatocyte growth factor and antibodies to HLA and MICA antigens in heart transplant recipients. Tissue Antigens. 2010 Nov;76(5):380-6. IF = 2.33

5. *Pavlova Y*, Viklicky O, Slatinska J, Bürgelova M, Süsal C, Skibova J, Honsová E, Striz I, Kolesar L, Slavcev A. Soluble CD30 and Hepatocyte growth factor as predictive markers of antibody-mediated rejection of the kidney allograft. Transpl Immunol. 2011 Jul;25(1):72-6. IF = 1.459

Comment to article 1 and 2

Pavlova Y, Kolesar L, Striz I, Jabor A, Slavcev A. Distribution of KIR genes in the Czech population. Int J Immunogenet. 2008 Feb;35(1):57-61.

Hollenbach JA, Meenagh A, Sleator C, Alaez C, Bengoche M, Canossi A, Contreras G, Creary L, Evseeva I, Gorodezky C, Hardie RA, Karlsen TH, Lie B, Luo M, Martinetti M, Navarette C, de Oliveira DC, Ozzella G, Pasi A, **Pavlova E**, Pinto S, Porto LC, Santos P, Slavcev A, Srinak D, Tavoularis S, Tonks S, Trachtenberg E, Vejbaesya S, Middleton D. Report from the killer immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA. Tissue Antigens. 2010 Jul;76(1):9-17.

Killer immunoglobulin-like receptors are a family of surface molecules that regulate NK cells and some subsets of cytotoxic T lymphocytes through interaction with ligands which are mainly HLA class I molecules. In case inhibiting ligands for KIRs are missing or their expression is too low, the NK cell is activated and kills the target cell. KIRs play an important role in anti-viral and anti-cancer immunity. Recently it has been suggested that incompatibility between KIR and HLA repertoire of the donor and recipient may be a risk factor for organ transplant rejection. Our study was the first aimed at investigating the KIR and KIR ligand repertoire in the Czech population. Although the KIR repertoire is not yet taken into consideration as a matching factor in donor / recipient pair selection, it may be a significant player in post-transplant immune reactions.

In the studied cohort of 125 healthy Czech individuals, we found 38 unique genotypes with a significant prevalence of A-haplotype homozygosity (over 25% of population) and demonstrated a strong similarity of the KIR repertoire of Czech population with other Caucasian populations, such as German and Polish. Meanwhile, the most profound differences were observed between Czech and Asian, Afro-Caribbean, North Indian populations. KIR ligands have been also analyzed and we detected the prevalence of C1/C2 heterozygosity (43%) and C1 homozygosity (41%) over the C2 heterozygosity (16%). Over 95% of individuals from the analyzed panel carried at least one inhibitory KIR for the corresponding HLA-C group found in the genotype.

This study analyses KIR and KIR-ligands diversity within a healthy Czech population and its' differences with other populations and ethnic groups. These results may be used in further comparative and associative studies aimed at the role of KIR in transplantation and other clinical disciplines. They should also be taken into account when extrapolating data from such studies on the Czech population.

Comment to article 3

Slavcev A, Honsova E, Lodererova A, **Pavlova Y**, Sajdlova H, Vitko S, Skibova J, Striz I, Viklicky O. Soluble CD30 in patients with antibody-mediated rejection of the kidney allograft. Transpl Immunol. 2007 Jul;18(1):22-7.

Antibody-mediated rejection remains one of the principal problems in organ transplantation, endangering the graft shortly after transplantation as well as in long-time perspective. Recently, there has been growing evidence in the literature that elevated a pre- and post-transplant concentration of the sCD30 molecule in kidney recipients' sera is a risk factor for acute kidney allograft rejection and impaired graft survival. CD30 is expressed on activated CD8+ and CD4+ lymphocytes stimulating B cells and activating Th2 responses. Therefore, it was suggested that sCD30 might be especially helpful in predicting the occurrence of AMR. The aim of our study was to test this hypothesis.

We tested a group of sixty-two kidney transplant recipients with and without AMR signs for sCD30 levels before and after transplantation.

Although pre-transplant sCD30 concentrations tended to be higher in patients who later experienced AMR than in those who had only CR (80.02±54.4 U/ml vs. 64.0±25.3U/ml), this result did not reach statistical significance. On the other hand, our study revealed that low pre-transplant sCD30 levels determined as concentrations below a conventional cut-off (<100 U/ml) may reliably distinguish patients who are at a low risk of C4d positive AMR within first post-transplant year (specificity was 82% in non- de novo DSA producers and 86% in de novo DSA producers).

Results of this work demonstrate that neither pre-transplant nor post-transplant sCD30 may be used as a stand-alone method for C4d positive AMR prediction, but sCD30 remains a useful parameter which allows to distinguish patients who are at low risk of AMR.

Comment to article 4

Pavlova YA, Malek I, Honsova E, Netuka I, Sochman J, Lodererova A, Kolesar L, Striz I, Skibova J, Slavcev A. Hepatocyte growth factor and antibodies to HLA and MICA antigens in heart transplant recipients. Tissue Antigens. 2010 Nov;76(5):380-6.

Retrospective studies demonstrated that both pre-transplant DSA and HLA incompatibility significantly influence the incidence of acute heart allograft rejection and survival. At the same time, MICA antibodies and HGF are relatively newly discovered factors and their possible impact on acute heart rejection incidence is as yet unclear. Our goal was therefore to analyze the combined effect of HLA and MICA antibodies and HGF concentrations on the incidence of acute graft rejection within the first six months after transplantation and the possible interplay between them.

None of the three factors – HLA antibody, MICA antibody and HGF – appeared to be directly associated with cellular rejection incidence in our cohort. On the other hand, our study revealed that the simultaneous presence of both HLA class I and II DSA before transplantation was associated with increased incidence of AMR ($p=0.025$).

An important finding was that elevated HGF production was associated with HLA antibody presence before and after transplantation ($p=0.022$ and $p=0.00029$, respectively). Interestingly, the highest HGF levels were observed in both class I and class II positive patients. In only class I positive patients HGF levels were lower than in the class I and II positive group, but still significantly exceeded HGF levels in patients without HLA antibody production. Even though after transplantation HGF concentrations significantly decreased in all patients, the strong correlation between post-transplant antibody presence and higher HGF levels persisted. We suppose that HGF elevation potentially may be a sign of activation of reparatory and proliferative mechanisms in response to endothelium injury caused by HLA-specific antibodies. It is known that the development of ChR is based on damage-repair-damage mechanism;therefore, elevated HGF might possibly help to identify those patients who are a-priori predisposed to more active repair and proliferative reactions. We suggest that we cannot exclude HGF from the list of factors possibly connected to rejection and further studies are needed to clarify its role in heart allograft outcome.

Comment to article 5

Pavlova Y, Viklicky O, Slatinska J, Bürgelova M, Süsal C, Skibova J, Honsová E, Striz I, Kolesar L, Slavcev A. Soluble CD30 and Hepatocyte growth factor as predictive markers of antibody-mediated rejection of the kidney allograft. Transpl Immunol. 2011 Jul;25(1):72-6.

Our earlier findings as well as results of other groups demonstrated that HLA-specific antibodies and sCD30 are predictive for AMR incidence. Nevertheless, there are only a few studies dedicated to the combined effect of these factors on kidney allograft rejection and loss. On the other hand, there is still little known about the role of HGF in kidney transplantation although results obtained so far in animal models are promising. For that reason, , in this study, we aimed at evaluation of HLA antibodies, elevated sCD30 and HGF in combination and separately as risk factors of the allograft loss and AMR incidence within two years after kidney transplantation.

In our cohort (n=205), the incidence of AMR was significantly higher in first- and re-transplant patients who simultaneously produced both HLA class I and II antibodies ($p<0.0001$). An intriguing finding was that while elevated pre-transplant sCD30 alone appeared to be predictive for AMR free survival only in re-transplanted patients, in the total cohort (first and re-transplant) patients who were positive for HLA antibody and had elevated sCD30 demonstrated the lowest AMR-free survival compared with patients with present antibodies but low sCD30 and patients free of antibody production ($p<0.0001$). Surprisingly, we observed a significantly lower AMR-free survival in patients with elevated sCD30 and low pre-transplant (HGF $\leq$500pg/ml; $p<0.0001$), while in patients with high HGF and high sCD30, the incidence of AMR was the same as in patients with low sCD30 concentrations. This may possibly mean that endogenous HGF in accordance with its described capability to protect tissues from oxidative and toxic injury may also have some kind of a protective and anti-AMR effect on the allograft.

We conclude that kidney graft recipients who produce both HLA class I and II antibodies before transplantation are at a significantly higher risk of AMR within first two years after transplantation. High sCD30 alone is unlikely to predict AMR but it unfavorably increases AMR incidence in the presence of HLA antibodies. Elevated HGF, while being not predictive for AMR incidence as a stand-alone test, might have an AMR-protective effect in patients with high sCD30.

Discussion

1. KIR gene repertoire is very diverse and varies within different populations as well as between them. Our study revealed 38 unique genotypes in the Czech population with the most frequent genotype produced by combination of two A-group haplotypes and thus bearing only one activating KIR, which was 2DS4, but various combinations of inhibiting receptors. This fact corresponds with the earlier findings describing A-haplotypes as the most frequently occurring across Caucasian populations (Rajalingam, 2008; Middleton & Gonzelez, 2010). Interestingly, in a considerable number of A haplotypes, the only activating K2DS4 gene bears a deletion and consequently this receptor is not expressed in a significant number of individuals (Rajalingam, 2008) which means that possibly a significant part of Czech population does not express any short-tailed KIR. As for further genotypes detected in the Czech population, the most profound differences in KIR gene representation was detected between Czech and Asian, Afro-Caribbean, North Indian populations, while German and other Caucasian populations (Italian, Greek, Polish) demonstrated a strong similarity with the Czechs (Pavlova et al., 2008; Hollenbach et al., 2010). This suggests that the results of research focused on the role of KIRs in transplantation performed in these populations may be to some extent applicable to our population and may suggest directions for such analyses in a Czech cohort.

As for the KIR ligand repertoire, we found that 16% of Czech individuals were homozygous for the HLA-C2 group and 43% were heterozygous (HLA C1/C2). Some studies revealed a positive effect of HLA-C2 allele in donors genotype on kidney and liver allograft survival (Kunert et al., 2007; Hanvesakul et al., 2008; López-Alvarez et al., 2009), which may suggest that approximately 59% of organ donors in Czech Republic potentially bear a “protective” genotype, while 41% of the donor cohort do not.

In our preliminary retrospective study of KIR genotypes in 150 heart transplant recipients, we observed that patients having the KIR 2DS5 gene in the KIR genotype appeared to be predisposed to heart transplant failure due to severe cellular rejection ($p=0.036$) while other non-framework KIR genes were not significantly associated with graft rejection (Kolesar et al., 2009). This study was presented as a poster and these results need a more detailed analysis with respect to recipients’ HLA-C genotype groups and donors’ KIR and HLA genotypes because of the high complexity of KIR and their ligand system.

In conclusion, KIR and their ligand variability between different populations should be taken into consideration when designing studies aimed at role of KIRs in transplantation and it should

also be regarded when extrapolating results of such studies on the Czech population. The extremely high variety of KIR and KIR-ligand haplotypes complicates the design and analysis of these studies due to limited number of pairs with particular KIR-HLA combination and poor KIR-HLA matching in retrospectively studied cohorts.

2. Among multiple factors that influence solid organ transplantation outcome, HLA specific antibodies are probably the best investigated. For decades, a CDC cross-matching test has been used to avoid hyperacute rejection in kidney recipients with preformed antibodies (Patel & Terasaki, 1969). Nonetheless, not all patients with negative pre-transplant CDC are clear from HLA antibody. They may produce them in low concentrations undetectable by CDC; cross-reactive antibody may have lower specificity and affinity to donors HLA, insufficient for turning CDC test positive; and last, but not least, these may be non-complement-binding antibodies. Consequently, our aim was to evaluate clinical relevance of pre- and post-transplant HLA antibodies as detected by FCXM, ELISA and/or Luminex assays.

In our studies, we found a strong correlation between C4d positive humoral rejection and the presence of antibodies either detected by FCXM or by solid phase assays (Slavcev et al., 2007; Pavlova et al., 2010; Pavlova et al., 2011). FCXM cross-match was more sensitive and specific in HLA antibody detection than CDC and its pre-transplant positivity was predictive for C4d positive AMR (p=0.046) (Slavcev et al., 2007). This finding is in correspondence with other works suggesting FCXM to be a reliable, clinically relevant method of DSA detection (Amico et al., 2008; Couzi et al., 2011).

In our later study, involving wider cohort of kidney transplant recipients (n=205), we investigated the separate effect of class I and class II antibodies as detected by ELISA on AMR. We observed that the simultaneous presence of HLA class I and II antibodies significantly decreased AMR-free survival (OR=12.87; CI 4.24-39.26). This finding is in correspondence with earlier reports about significant deteriorating effect of combined HLA antibody to class I and II on the post-transplant course in kidney transplantation (Süsal & Opelz, 2002; Süsal et al., 2009). These results are also in line with a recent work of Seyhun et al., who reported that HLA class II and MICA antibodies are important predictors of graft failure only when present together with HLA class I antibodies (Seyhun et al., 2012).

Interestingly, we also observed a trend of higher incidence of HLA class I, but not class II antibodies in patients who irreversibly rejected the graft in comparison with those who had functioning graft by the end of the 2-year follow-up period (45% vs. 28%; p=0.082). This trend is

in concordance with earlier studies that revealed correlation of lower allograft survival with HLA class I antibody presence (Süsal & Opelz, 2004; Süsal et al., 2009; Riethmüller et al., 2010). Such correlation might have two mechanisms: 1. HLA class I antibodies activate complement and recruit immune cells, thus causing acute AMR episodes; 2. Binding of HLA class I antibodies on the endothelial cells surface stimulates proliferation, leading to the intimal thickening and further changes typical for the ChR (Cecka et al., 2005; Zhang et al., 2012). Nonetheless, in our study, a trend towards the linkage between class I antibody and impaired graft survival did not reach statistical significance. This might be because the follow-up period was limited to two years while in many cases signs of chronic rejection and especially irreversible graft failure caused by it may occur later. The other possible reason is that in some positive sera, even though present, antibody titer may be below clinically relevant level and, in this case, may even increase resistance of the allograft to antibody damage (Cecka et al., 2005).

In the study, which is focused on AMR and CR in heart transplant recipients, we used the Luminex single antigen (SA) assay for detection of HLA antibodies. It is still a matter of debate whether Luminex SA alone is a reliable tool for the evaluation of the risk of rejection. Some recent studies claim that oversensitivity of this method might complicate discrimination between clinically relevant and irrelevant HLA antibodies. Vlad et al. reported that they did not find strong correlation between SA detected antibodies and acute kidney rejection episodes, while Süsal et al. also observed lack of correlation between antibodies detected exclusively by Luminex SA and kidney allograft loss (Vlad et al., 2009; Süsal et al., 2011).

In heart transplantation, most reports are focused on C4d binding antibodies detectable by Luminex and on a graft loss rather than on acute rejection incidence (Smith et al., 2007; Irving et al., 2011). The question we addressed in our study was whether pre-transplant DSA as detected by Luminex SA assay irrespectively to their C4d fixing capabilities influence the occurrence of early post-transplant rejection.

We observed a strong correlation between combined HLA class I and II DSA production and higher AMR incidence in heart transplant recipients ($p=0.025$) while CR was not influenced by the presence of HLA antibody. The role of NDSA remained marginal and patients with NDSA had nearly the same AMR-free survival as patients without antibodies (92% vs. 90%). This finding is in line with the earlier report of Stastny et al. who found a correlation between AMR incidence and combined pre-transplant production of class I and II DSA as determined by Luminex SA in cardiac allograft recipients while NDSA appeared to be irrelevant (Stastny et al., 2007). Interestingly, out of 21 patients with DSA before transplantation, 14 became DSA-negative and

remained clear from DSA during follow-up period. This might be, on one hand, due to accumulation of antibodies in the allograft and, on the other hand, due to the application of immunosuppressive agents, especially Mycophenolate mofetil (MMF), which is known to reduce antibody production (Lederer et al., 2005). Indeed, among 12 patients receiving MMF, 10 became HLA antibody negative post-transplant (80%), while out of 9 patients without MMF only 4 turned negative (44%) (p=0.03).

In conclusion, preformed HLA antibodies as detected by FCXM, ELISA and/or Luminex (xMap) are predictive for the incidence of AMR while their role in acute cellular rejection appears to be marginal. The combined presence of class I and II antibodies is the most unfavorable for AMR-free survival in kidney and in heart transplant recipients. Donor-specificity of HLA antibodies should be taken into consideration for the more precise rejection risk evaluation.

3. MICA antibodies are a relatively newly discovered factor which apparently may influence acute and chronic allograft rejection and loss. At present, there are only few studies focused on pre- and post-transplant MICA and their clinical relevance in heart transplantation. Our aim was therefore to investigate the role of MICA antibodies as defined by Luminex SA in early AMR and CR incidence in heart allograft recipients. We did not observe any correlation between AMR and/or CR incidence and the production of MICA antibodies before transplantation. This finding is in line with the report of Smith et al. who found pre-transplant MICA to be irrelevant for predicting acute rejection episodes within the first post-transplant year as well as for evaluating the risk of allograft loss (Smith et al., 2009).

Interestingly, in our cohort, post-transplant de novo MICA antibody production was more frequent than de novo HLA antibody production (n=11 vs. n=3). We suppose that this is due to the fact that MICA antigens are stress-inducible and their expression on the graft endothelial cells raises post-transplant causing MICA-specific allosensitization.

Post-transplant MICA antibodies in the studied cohort did not influence the incidence of CR, whereas AMR tended to be more frequent in patients with de novo MICA antibodies. Although this trend did not reach statistical significance (p=0.063), it is in line with earlier reported post-transplant MICA correlation with increased acute rejection incidence and decreased heart transplant survival (Suárez-Alvarez et al., 2007; Kauke et al., 2009; Nath et al., 2010).

Our study showed that, in heart transplant recipients, pre-transplant MICA antibody production has no significant predictive value for AMR as well as for the incidence of acute CR . Post-transplant MICA production however tends to be more frequent in heart recipients who are at the

higher risk of AMR. Further studies including analysis of anti-donor-specificity of MICA antibodies should be performed to clarify the role of MICA antibodies in heart allograft course.

4. Based on previous studies proposing sCD30 as even more reliable predictor of rejection than pre-transplant PRA (Cinti et al., 2005) our study was intended to evaluate sCD30 as a possible predictive marker of AMR. We observed that while elevated pre-transplant PRA detected by CDC strongly correlated with AMR incidence, the association between elevated sCD30 and AMR was not straight-forward (Slavcev et al., 2005; Pavlova et al., 2011). Along with further investigators (Rajakariar et al., 2005; Kim et al., 2006); we did not find a significant correlation between high pre-transplant sCD30 and C4d positivity in biopsies. We also did not find sCD30 to be predictive for de novo antibody production which is in agreement with the data of Weimer et al. (Weimer at al., 2006) who also could not find such correlation. In re-transplant patients, however, we observed a trend towards lower AMR-free survival in patients with sCD30 $\geq$ 100 U/ml before transplantation ($p=0.051$) which suggests that sCD30 might be informative about the individual immune reactivity in immunologically risky patients (Pavlova et al., 2011).

Another question we addressed was whether the combined analysis of HLA antibodies and sCD30 production could be helpful in estimating the risk of development of AMR.

In the study performed by our group in 2007, pre-transplant measurement of sCD30 in patients who produced de novo antibodies after transplantation demonstrated high specificity for C4d incidence, i.e. likelihood that if sCD30 is negative, C4d deposits will not occur. This finding suggested that negative sCD30 might be useful for identifying patients who are at a lower risk of development of C4d+ AMR even though producing DSA (Slavcev et al., 2007). Nevertheless, in this study p did not reach significant level.

Results of our later study revealed that patients positive for HLA antibody either class I, II or both, as determined by ELISA, and simultaneously producing high sCD30 ($\geq$ 100 U/ml) before transplantation had the lowest AMR-free survival in comparison with patients free of antibody production and/or sCD30 elevation ($p<0.0001$) (Pavlova et al., 2011). This is in correspondence with finding of Vaidya et al., who reported significantly higher AMR incidence in patients positive for both antibody and elevated sCD30 in comparison with those who produced low sCD30 and did not produced HLA antibody (Vaidya et al., 2006). Unlike Vaidya et al., we included into the analysis patients with high sCD30 and lacking antibody as well as antibody-producers with low sCD30 and, according to Kaplan-Mayer's analysis, these two groups had an AMR-free survival rate comparable with patients free from antibody production and elevated sCD30 which suggests

that elevated sCD30 and antibody production is likely to have a synergic effect on the incidence of AMR.

While a number of studies report that elevated sCD30 and HLA antibody production have synergistic (Rodriguez et al., 2007; Süsal et al., 2011) or additive (Wang et al., 2012) effect on the kidney allograft loss, our work suggests possible explanation to this effect by demonstrating correlation between elevated sCD30, HLA antibody and increased AMR incidence.

In conclusion, we did not confirm that pre- and post-transplant sCD30 measurement may be sufficient for the prediction of AMR development in kidney allograft recipients. Nevertheless, it should be taken into consideration while evaluating the probability of AMR development in individuals with high immunological risk, especially those producing HLA antibodies.

5. Hepatocyte growth factor has been studied for over a decade as a factor participating in liver, kidney, lung, muscle and further tissue development and regeneration. Recently, it has also been suggested as a possible predictor of acute rejection incidence and/or a marker of the on-going rejection and this suggestion was confirmed in animal models (Yoshimura et al., 2002), but only a few studies so far have analyzed HGF in human organ recipients. Therefore, we investigated the role of pre- and post-transplant HGF in kidney and heart rejection alone and in combination with HLA antibody presence and sCD30 production.

In our cohort of heart transplant recipients, we did not observe direct correlation of pre- and post-transplant HGF levels and AMR and CR incidence. Moreover, HGF measurement performed at the time of ongoing cellular rejection did not differ from HGF concentrations measured when patients were free of rejection. Thus, we could not confirm the finding of Zwirska-Korczala et al. who reported a significant post-transplant HGF elevation in patients with acute rejection (Zwirska-Korczala et al. 2005). This contradiction might be due to differences in patient demographic characteristics, because Zwirska-Korczala et al. included only male patients into the studied cohort and excluded patients with clinical symptoms of heart failure and/or systolic or diastolic dysfunction of the left ventricle while we did not apply such limitations.

Our study was the first analyzing HGF concentrations with respect to antibody presence in heart transplant recipients and revealed that patients producing HLA class I or combined class I and II antibodies before transplantation had a significantly higher HGF concentrations than those free of antibody production (2072±1396 pg/ml vs. 1149±795 pg/ml; p=0.002). This association was even stronger in the post-transplant period (1754±893 pg/ml vs. 607±394pg/ml; p=0.00029). We suggest that this could be explained by the capability of HLA class I antibodies to induce damage

to endothelial cells and induce growth factors production (Cai & Terasaki, 2005; Jindra et al., 2008). Interestingly, there was no correlation between HGF and MICA antibody production before and after transplantation, which probably suggests that the mechanisms involved in MICA and HLA antibody interaction with endothelial cells differ as well as differing in their damaging and activating potential.

In kidney transplant recipients, we observed a significant increase in HGF concentration within first two weeks after transplantation which is in line with earlier reports who described the same post-transplant dynamics in HGF concentration. Such elevation is presumably a protective reaction caused by allograft hypoxic stress and intraoperative tissue injury (Aharinejad et al., 2004; Kwiatkovska et al., 2010).

In our cohort of kidney transplant recipients, we did not observe a correlation between HLA class I production and HGF concentrations. Moreover, HGF was even significantly lower in patients producing HLA class II antibodies before transplantation and 6 months post-transplant ($p=0.001$ and $p=0.05$ respectively). The mechanism of a possible linkage between HGF and HLA class II production has not been described to date.

The number of HLA class II producers was significantly higher among re-transplant patients than among first transplant patients (38% vs. 4%; $p<0.0001$). At the same time, re-transplant patients with HLA class II antibodies tended to have lower HGF concentrations (314±155 pg/ml) than those who received their first transplant (422±172 pg/ml). Taking into consideration that HGF was reported to have a significant tissue-protective and rejection-suppressive effect (Yamaura et al., 2004; Franquesa et al., 2005), one might speculate that low endogenous HGF could be to some extent a part of an immunologically “risky” phenotype possibly influencing graft loss, which, in turn, increases the probability of HLA class II antibody occurrence. This suggestion is based on indirect prerequisites and needs further investigation to be confirmed or rejected.

We cannot also exclude the influence of further factors which were not regarded in this analysis but are known to influence HGF concentrations such as peritoneal dialysis and heparin intake.

Interestingly, the combined analysis of HGF and sCD30 production revealed that the lowest AMR-free survival was in patients with low HGF and high sCD30 while in all other groups (low HGF and low sCD30; high HGF and high sCD30; high HGF and low sCD30) there was no significant difference in the AMR-free allograft survival ($p<0.0001$). This suggests that elevated HGF (over 500 pg/ml) might be rejection-protective even in patients with unfavorable immunological status. This observation is in line with results obtained in animal models (Yamaura

et al., 2004; Franquesa et al., 2005). The effect of high sCD30 and low endogenous HGF appears to be synergic and the analysis of both factors could help identify patients at an increased risk of acute rejection.

All in all, our studies revealed no direct correlation between HGF concentrations, AMR or CR incidence and graft survival in heart and kidney transplant recipients. Nevertheless, apparently HGF is involved in post-transplant graft injury and repair processes and may be useful when analyzed together with further molecular factors. The observed correlation between HLA antibody production and elevated HGF concentrations in heart transplantation requires further investigation focused not only on acute rejection episodes but also on ChR incidence and graft loss. We also suggest that possible protective effect of endogenous HGF in kidney graft recipients should be investigated as it could possibly explain some cases of rejection-free survival and normal graft function in immunologically compromised patients.

Conclusions

1. Ethnicity plays a significant role in KIR gene distribution and the particular KIR haplotype representation which must be taken into consideration when extrapolating data from studies performed on different ethnic groups or conducting comparative studies. KIR repertoire of the healthy Czech population is similar with other Caucasian populations. The extremely high variety of KIR and KIR-ligand haplotypes complicates clinical studies due to the limited number of pairs with particular KIR-HLA combination and poor KIR-HLA matching in retrospectively studied cohorts.

2. Preformed HLA antibodies, as detected by FCXM, ELISA and/or Luminex (xMap), are predictive for the incidence of AMR while their role in acute cellular rejection needs further investigation. Combined presence of HLA class I and II antibodies is the most unfavorable for the C4d positive antibody-mediated rejection incidence in kidney and heart transplantation. Donor-specificity of HLA antibodies should be taken into consideration for more precise rejection risk evaluation.

3. Our study did not confirm that pre- and/or post-transplant sCD30 level measurement may be used as a stand-alone prognostic parameter for AMR in kidney transplant recipients. However, when analyzed together with HLA antibody production, sCD30 is helpful in detection of patients who are at a higher risk of AMR.

4. Pre-transplant MICA antibodies appeared to have marginal effect on early AMR and CR in heart transplant recipients while post-transplant MICA antibody presence tends to influence the incidence of AMR. Further studies, including larger cohorts and performed with respect to donor-specificity of MICA antibodies, should be performed to clarify the role of MICA in heart allograft course.

5. HGF before and after transplantation is not directly correlated with AMR or CR either in heart or in kidney allograft recipients. Nonetheless, HGF is apparently involved in post-transplant graft injury and repair processes and may be useful when analyzed together with other molecular factors such as HLA antibody production and sCD30 concentrations. The observed correlation between HLA antibody production and elevated HGF concentrations in heart transplant recipients requires further studies focused not only on acute but also on chronic rejection incidence and graft loss. We also suggest that the possible protective effect of endogenous HGF in kidney transplants needs further investigation.

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