

KEYNOTE PRESENTATION

00-01

The long-term impact of fetal-maternal cell traffic during pregnancy

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Some cells traffic between fetus and mother during pregnancy and low levels persist in respective hosts years later. Microchimerism refers to an individual harboring small numbers of cells (or DNA) derived from another genetically distinct individual. Chronic graft-versus-host disease is an iatrogenic form of chimerism that occurs as a complication of hematopoietic cell transplantation and has clinical similarities to some autoimmune diseases. The HLA-relationship of a donor and recipient is of central importance in graft-versus-host disease. Considering these observations together led to the hypothesis that naturally acquired microchimerism from pregnancy and the HLA relationship among different cell populations impacts long-term health

and autoimmune disease risk. The hypothesis is applicable to children, men and women who have never been pregnant because transfer of maternal cells, or cells from a twin, or a blood transfusion can result in microchimerism. Microchimerism could also potentially derive from an older sibling passed via the maternal circulation in a subsequent pregnancy. Studies have investigated microchimerism in systemic sclerosis (scleroderma), primary biliary cirrhosis, Sjögrens syndrome, polymorphic eruption of pregnancy, myositis, thyroid disease and systemic lupus. Results support a potential role in some diseases. However, microchimerism is commonly found in healthy individuals. Thus it is likely that naturally acquired microchimerism can have both beneficial and detrimental effects on the host. Microchimeric cells that bear tissue specific antigens have recently been identified (e.g. cardiac myocytes) suggesting tissue-specific microchimeric cells could be the target of an "autoimmune" response or, alternatively, that microchimeric cells traffic to sites of tissue damage and contribute to tissue repair.

IMMUNOGENETICS IN REPRODUCTION

Plenary lecture

01-01

Ontogeny of MHC during development, in mice and humans

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The sequencing of the mouse and human genome has emphasized the need for identifying the proportions of the genomes under selection pressures, in particular the number of protein-coding genes and their ontogenies and the evolutionary expansion of gene families related to reproduction and development. In the laboratory mouse (*Mus musculus*), MHC Class I mRNAs encoding the heavy chain of H-2 D^b and Q7/9 encoding Qa2 molecules are expressed at one-cell stage of embryonic development. The non-polymorphic MHC Class I associated molecule β_2 -microglobulin is detected at the eight-cell stage. The intracytoplasmic peptide transporters TAP1 -but not TAP2- were first detected at the blastocyst stage. These data suggest that MHC Class I products are synthesised soon after conception prior to implantation. Thus, we and others have conclusively shown that MHC Class I mRNA encoding the heavy chain is present in the mouse zygote from the late 1-cell stage. This expression involves zygote derived gene transcription, since we detected mRNA of the paternal origin allele in crosses between mice of differing H-2 haplotypes. The requirement for MHC Class I expression, in the correct quantitative levels, early in development, has been emphasised by experiments reported by Jaffe *et al* 1992, who have shown that mouse embryos over-expressing the Class I product, H-2D^d fail to develop beyond mid-gestation. Based on the above observations, we have proposed the hypothesis that a quantitative expression of MHC Class I transcription is required for optimal embryonic growth and this provides the correct homeostatic gene profile for development. We have also observed an impaired breeding phenotype in transgenic mice devoid of β_2 -microglobulin, suggesting a role for this molecule in breeding rates. The picture in humans is similar; MHC Class I HLA-G transcripts have been reported on blastocysts, some oocytes and preimplan-

tation embryos. Isoforms of HLA-G are predominantly expressed as soluble proteins into the embryo micro-environment. Overall, these data suggests that MHC class I molecules have a role in reproduction and probably in stage-specific embryonic development.

Short oral presentations

01-02

Intron-4 containing HLA-G is not detectable in trophoblast conditioned mediaA Blaschitz¹, H Juch¹, A Volz², H Hutter¹, G Dohr¹¹Institute of Cell Biology, Histology and Embryology, Center of Molecular Medicine, Medical University of Graz, Austria; ²Institut für Immunogenetik, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin, Germany

In the context of implantation and pregnancy several immunomodulating functions have been attributed to HLA-G. Increasing attention is now being addressed to the actively secreted soluble isoforms, since they might have a systemic function or could be useful as diagnostic tools. However, the cellular source of secretion, even during pregnancy, where HLA-G expression level is known to be highest, is still under debate. To elucidate conflicting reports, published so far, we investigated the isoform distribution in human first trimester and term placentas *in situ* and *in vitro*. Results obtained by applying immunohistochemistry, Western blot, ELISA and RT-PCR show that all of the $\alpha 1$ domain-containing HLA-G isoforms are restrictedly expressed in the extravillous cytotrophoblasts and very few first trimester syncytiotrophoblasts, which directly cover cell columns. Western blot analysis demonstrates that trophoblast expresses only the HLA-G1 isoform. However, on mRNA-level we detected extremely low levels of HLA-G5, -G6 in term placenta tissue samples and in isolated first trimester trophoblasts. Media conditioned by primary first trimester trophoblasts and the chorion laeve-derived trophoblastic cell line AC1-M59 solely contained HLA-G1 fragments. Our data provide substantial evidence that none of the intron 4-containing isoforms, the so called actively secreted, soluble HLA-G5 or -G6 are produced by human trophoblasts.

01-03

Detection of HLA-E and HLA-G expression in human placental tissue

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Nonclassical MHC class Ib human leukocyte antigen E (HLA-E) and HLA-G molecules differ from classical ones by specific patterns of transcription, protein expression, and immunotolerant functions. HLA-G can be expressed as four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) proteins upon alternative splicing of its primary transcript. In this study, we used a set of monoclonal antibodies (mAbs) called HCA2, 4H84, MEM-G/1, -G/9, MEM-E/2, and -E/6 recognizing HLA-G or HLA-E. The patterns of reactivity of these mAbs were analyzed on transfected cells by Western blotting and immunocytochemistry. MEM-G/1 recognizes (similar to the 4H84 mAb) the denatured HLA-G heavy chain of all isoforms. MEM-G/9 mAb react exclusively with native HLA-G1 molecules. MEM-E/2 and -E/6 mAbs bind the denatured and cell surface HLA-E molecules, respectively. These mAbs were then used to analyze the expression of HLA-G and HLA-E on cryo-preserved and paraffin-embedded serial sections of placental tissue. Single and double-immunolabeling with the respective mAbs revealed the presence of HLA-G and HLA-E in extravillous cytotrophoblast. In comparison, however, the levels of HLA-E expression seem to be much lower than those of HLA-G. In addition, we found a strong expression of HLA-E in endothelial cells using mAb MEM-E/2. The presence of HLA-G and HLA-E in specific cell populations of the placenta suggest an interacting functional role of these molecules in maternal-placental immune recognition.

Plenary lectures

01-04

Transcriptional profiling of uterine and blood NK cells from syngeneic and allogeneic pregnancy

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NK cells are the predominant lymphoid population in the uterus in both human and rodent pregnancy. Their exact function is still unclear, but evidence supports a role in regulating placental development and modifying uterine vasculature to provide adequate blood supply to the fetus.

Objectives: The objectives were 1) to compare gene expression in uNK cells and blood NK cells, in order to detect uNK-specific genes and reveal differences that might clarify the function of this population; 2) to compare uNK gene expression in syngeneic and allogeneic pregnancies, in search of differential gene expression induced by the recognition of paternal antigens.

Methods: Antibody-coated beads were used to isolate rat gp42+ uNK cells and NKR.P1+ CD3- blood NK cells, from which total RNA was isolated. cDNA was generated and amplified using the SMARTTM system (Clontech, BD Biosciences) and competitively hybridised to a new rat 15K cDNA microarray, recently produced by the Wellcome Trust Sanger Institute using clones from the University of Iowa Rat EST Project. The SAM: Significance Analysis for Microarrays tool (Stanford University) was used for significance testing of Lowess-normalised data. Results for selected genes were verified with Real Time PCR (19/20 confirmed).

Results: 1) Over 1000 genes were consistently differentially expressed between blood and uterine NK cells; 107 genes exhibited a difference in expression level of 10-fold or above between the two populations. This profile provides insight into their functional differences. 2) None of the genes on the array showed significant differential expression between uNK cells from syngeneic and allogeneic pregnancies.

01-05

Mate selection, olfactory receptors, and the MHC

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In animals, it is typically the female that selects a mating partner. This decision can occur before,

during, and after copulation, and in vertebrates, polymorphic products of the major histocompatibility complex (*MHC*) have a role in mate choice. *MHC* heterozygosity in the offspring might be favoured, since it would provide an advantage in dealing with infections, while rare *MHC* alleles might be selected to prevent pathogen evasion of host immune responses or autoimmunity. During the initial encounter of potential partners, *MHC*-dependent odours influence sexual selection in several groups of vertebrates, including humans. We point out that various types of chemoreceptors within the main olfactory epithelium and the vomeronasal organ serve to recognize these odours, and we review recent evidence for an involvement of peptides bound to *MHC* molecules in reproductive contexts in mice and fish. Furthermore, following copulation, *MHC* antigens or fragments derived from them might serve to guide spermatozoa towards the oocyte. Odorant receptors on the spermatozoal surface participate in sperm guidance, and we suggest how "cryptic" female choice might be exerted, resulting in fertilization by a spermatozoon which optimally complements the genetic contribution of the female to the embryo. Furthermore, prezygotic control within the fertilized oocyte could be influenced by *MHC*-encoded transcription factors, in analogy to mechanisms that participate in mate choice in fungi. In conclusion, to secure an optimal genetic constitution of the offspring in vertebrates, the partners have to overcome several barriers which might be influenced to various extents by the partners' *MHC* and polymorphic chemoreceptors.

01-06

The role of *MHC* class I genes in normal and cloned cattle pregnancy

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Major histocompatibility complex (*MHC*) class I genes encode highly polymorphic cell surface glycoproteins that bind and present antigenic peptides to cytotoxic ($CD8^+$) T cells. They also interact with receptors expressed on natural killer (NK) cells, which have an important role in monitoring cell status. *MHC* class I is thus a crucial component of both acquired and innate immunity. *MHC* class I expression is more complex in cattle than human, with at

least 6 rather than 3 classical class I genes expressed in various combinations on different haplotypes. The mammalian fetus is potentially at risk from the maternal immune response because it expresses polymorphic paternal antigens, including *MHC*. Different species appear to use different strategies to avoid this problem. In human, expression of non-classical, non-polymorphic *MHC* genes is seen, and in addition to their key role in regulating maternal immune responses they may also be important in other areas, for example successful implantation and embryo development. Relatively little work has been carried out on *MHC* expression in fetal and trophoblast tissue in cattle. This is an area of interest for the cattle breeding industry, because of falling fertility rates and concerns about the use of cloned animals. In addition, study and manipulation of cattle embryos may reveal insights that will prove important for human IVF research. Studies on term cattle placenta show little if any class I expression at the trophoblast cell surface, despite significant levels of mRNA for classical class I. There is also evidence for transcription of non-classical class I genes at this stage. There is evidence that levels of class I expression in early (pre-implantation) cattle embryos are linked to development rate. We are currently investigating the nature of this *MHC* expression, in both normal and cloned embryos.

01-07

Influence of *KIR* and *HLA-C* genes on reproductive success

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Trophoblast cells invade into the uterus during the first half of human pregnancy. The function of these cells is to tap into the maternal blood supply by invading and replacing the walls of the uterine arteries. This infiltration is normally tightly controlled but under-invasion may occur when the arteries are inadequately modified and as a result the fetoplacental unit becomes under-perfused. This is the primary defect in pre-eclampsia and intra-uterine growth restriction (IUGR). At the other extreme over-invasion by trophoblast can lead to uterine rupture and haemorrhage. As the trophoblast cells are allogeneic it is thought that an

important element in the balancing control mechanism is immunological. Indeed, there are several features of pre-eclampsia, such as partner specificity and increased incidence in oocyte donation pregnancies, which suggest an immunological basis to the disease. The invading trophoblast cells express a unique combination of MHC class I molecules, HLA-G, HLA-C and HLA-E. Of these three molecules only HLA-C is polymorphic and will differ in trophoblast from each pregnancy in the same women depending on the inherited paternally-derived HLA-C allotype. The classical, highly polymorphic transplantation antigens, HLA-DR, HLA-A and HLA-B are absent. On the maternal side the classical T and B lymphocytes of the adaptive immune system are sparse. Instead, the dominant population present are an unusual population of Natural Killer (NK) cells. We have studied the receptors expressed by uterine NK cells which can bind to trophoblast HLA class I molecules focussing on the polymorphic HLA-C. NK cells can discriminate between all HLA-C allotypes as one of two groups, C1 and C2. The NK receptors for HLA-C are members of the Killer Immunoglobulin Receptor (KIR) family and both activating and inhibitory receptors for HLA-C are found. The KIR gene system is also polymorphic particularly in the number of activating and inhibitory KIR found in different individuals. Our hypothesis is that certain combinations of maternal KIR genotypes and fetal HLA C1 and C2 groups may modulate NK cell function to provide optimal balanced trophoblast invasion. We have found that mothers lacking activating KIR are at a significantly increased risk of pre-eclampsia if combined with the presence of HLA-C2 group in the fetus. This indicates that both maternal and paternal innate immune genes contribute to the development of PE and reproductive failure.

01-08

Genetic and functional relationships between MHC and NK receptor genes and their products

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Over 7% of the genes in the human genome are involved in defence against infection (Kelley *et al.*

Genomics 2005, 85 503–11). Some of these genes have features consistent with this role. For example, many of them are polygenic and polymorphic. They tend to be clustered in the genome. Some are evolving rapidly, by allele or gene conversion. We are studying some of these immune system genes to determine their role in disease resistance. Our main focus includes the MHC and NK complexes as the extensive variation in both MHC and NK receptor loci may reflect continuous selection for resistance to pathogens (Kelley *et al.* Genomics 2005, 85 503–11; Trowsdale & Parham, Eur J Immunol 2004, 34 7–17; Stewart *et al.* Genome Res 2004, 14 1176–1187; Martin *et al.* J. Immunol. 2003, 171 2192–2195). And, since the genes are on different chromosomes it is expected that there may be epistatic interactions between products of alleles from class I and NK receptors. In collaboration with other groups, including Mary Carrington's laboratory at Frederick, we are obtaining evidence for these interactions in MHC class I-associated diseases.

01-09

Regulatory T cell function in transplantation

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After exposure to alloantigen *in vivo* and *in vitro*, alloantigen specific immunoregulatory activity is enriched in a population of CD4⁺ T cells that express high levels of CD25. *In vivo*, common mechanisms appear to underpin the activity of CD25⁺CD4⁺ Treg in both naive and manipulated adult hosts. However, the origin, allorecognition properties, and molecular basis for the suppressive activity of CD25⁺CD4⁺ T reg, as well as their relationship to other populations of regulatory cells that exist after transplantation, remain a matter of debate. We have investigated 3 potential routes by which CD25⁺CD4⁺ Treg could be generated in adult mice after pre-treatment with alloantigen in combination anti-CD4 therapy. In this model, we were able to show that the development of Treg was not dependent on the presence of a thymus and CD25⁺CD4⁺ Treg, generated following alloantigen in combination anti-CD4 therapy could be generated from CD25⁺CD4⁺ peripheral T cells to a regulatory phenotype. Exploration of

the functional activity of CD25⁺CD4⁺ Treg in this setting has revealed a role for IFN-gamma. The identification and characterisation of regulatory T cells (T reg) that can control immune responsiveness to alloantigens has opened up exciting opportunities for new therapies in transplantation. These observations may have important implications for the design of clinical protocols to induce allograft tolerance in adult recipients.

01-10

Transcriptional regulation of MHC genes

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The products of the major histocompatibility complex (MHC) class I and class II genes encode cell surface glycoproteins, which are involved in binding and presentation of antigenic peptides to the T cell receptor (TCR) of T lymphocytes. Besides their important role in antigen presentation, both classes of MHC molecules can also serve as signal transducing receptors. Essential for transcription of MHC class II genes is the class II transactivator (CIITA), encoded by the *MHC2TA* gene. CIITA is a co-activator that interacts with the MHC-enhanceosome, which assembles on the SXY-promoter module of MHC class II and accessory genes. This SXY module is also present in the promoters of MHC class I (with the exception of HLA-G) and of β_2 -m genes. The *MHC2TA* gene itself is tightly regulated at the transcriptional level. In humans at least three functional promoters have been identified. Of these promoters, type I is responsible for the constitutive expression of CIITA in dendritic cells, while type III is responsible for the constitutive expression of CIITA in B cells. Notably promoter type III is also employed by activated T cells. The type IV promoter is the principal interferon- γ responsive promoter and is employed in non-professional antigen presenting cells. Furthermore, in humans interferon- γ also activates the type III promoter, which is governed through an upstream interferon-gamma regulatory region. These individual *MHC2TA* promoters are activated by cell-type-specific and general DNA-binding factors that assemble on the various

MHC2TA promoters. Furthermore, recent studies have also indicated that *MHC2TA* gene transcription in different cell types is governed both by genetic and epigenetic mechanisms. In particular, both DNA and histone modifications play an important role in silencing of *MHC2TA* gene transcription in some types of cancer cells and in trophoblast cells, which correlates with their MHC class II-deficient phenotype.

Poster presentations

01-11

Plasmatic HLA-G during pregnancy: a mother affair?

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HLA-G plays an important role in tolerance during pregnancy by inhibiting the cytotoxicity of maternal NK cells. Trophoblasts express HLA-G, but other cells such as monocytes can also produce it. The aim of this work is to investigate plasma HLA-G during pregnancy. Blood was obtained monthly from volunteers during normal pregnancy. We analyzed plasma HLA-G levels by ELISA, and by flow cytometry HLA-G expression in monocytes incubated 48 hours with or without IFN- γ . Plasma HLA-G concentration in pregnant women was significantly higher ($p < 0.005$) than in non-pregnant. However, plasma HLA-G concentration has a rhythm during pregnancy: it increases until a maximum at the beginning of the second term ($p < 0.05$), then it decreases ($p < 0.05$) to the end of the second term, with another increase again during the third term. Stimulation of non-pregnant monocytes with IFN- γ increased slightly surface HLA-G expression (median after IFN- γ treatment: 6,6%; median control: 4,5%). The stimulation of monocytes from pregnant women with IFN- γ produced a higher HLA-G expression (median after IFN- γ treatment: 24,1%; median control: 6,2% $p < 0.05$). IFN- γ provoked a higher HLA-G expression in pregnant compared to non pregnant women ($p < 0.05$). As we have previously shown in plasma, HLA-G expression was higher at the end of first term of pregnancy. Taken together, these results suggest that the main source of HLA-G in plasma during pregnancy may be maternal.

01-12

Soluble HLA-G in the culture media of human preimplantation embryos from cleavage stages to blastocyst and embryos undergoing preimplantation genetic diagnosis

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Major Histocompatibility Complex (MHC) genes are transcribed during pre-implantation embryo development. In humans, HLA-G –a non-classical MHC Class I product- has been hypothesised to play an immunosuppressive role in fetal-maternal tolerance. Like other MHC Class I products, a membrane-bound and soluble HLA-G form are produced. Detectable levels of soluble HLA-G (sHLA-G) have been observed in the culture media of some human preimplantation embryos. The exact role of these molecules is not clear, however, sHLA-G has been correlated with faster cleavage and high implantation rates in embryos. In IVF the aim is to maximize implantation rates whilst minimizing the number of embryos transferred so reducing the incidence of multiple pregnancies. Thus measurement of sHLA-G could be a non-invasive biological marker for assessing embryo implantation potential. Embryos from patients undergoing IVF were cultured in 50 µl droplets of Vitrolife sequential medium under mineral oil. Embryos were transferred to fresh medium droplets daily and the supernatant frozen for sHLA-G analysis using a sHLA-G sandwich ELISA assay. Supernatant from the JEG-3 cell line (a human placental choriocarcinoma) was used to establish comparative correlates. The profiles of sHLA-G levels at different stages of pre-implantation development were annotated and will be presented according developmental stage, morphological quality, and chromosome complement for embryos undergoing genetic diagnosis.

01-13

Measurements and associations of HLA-G cell surface receptors on a human placental choriocarcinoma cell line: Application of single particle fluorescence imaging

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HLA-G is a non-classical class I antigen expressed on the placental cytotrophoblast associated with fetal allograft tolerance. Several aspects of HLA-G expression, including density, association and mechanism of action have not been elucidated. In order to unravel some of these issues we are applying a non-invasive single cell imaging approach. Single particle fluorescence imaging (SPFI) uses the high sensitivity of fluorescence to visualise individual molecules that have been selectively labelled with fluorescent particles. The images of particles are diffraction-limited spots that are analysed by fitting with a 2-D Gaussian function. This technique determines positional information and the spot intensities depend on whether they arise from one or more particles; this provides the basis for determining and quantifying self-association of cell surface receptors. For SPFI analysis, choriocarcinoma derived cells (JEG-3) were cultured in Lab-Tek micro-chambers and labelled with a specific HLA-G monoclonal antibody conjugated with phycoerythrin. In parallel with this approach HLA-G cell surface expression has been investigated using confocal microscopy and receptor quantification with flow cytometry. The techniques used in this work will be applied to investigate HLA-G expression in human pre-implantation embryos and elucidate the role of HLA-G in development.

CYTOKINES IN BASIC AND CLINICAL REPRODUCTIVE IMMUNOLOGY

Plenary lectures

02-01

NK cells and the Type1/Type 2 cytokine shift in human pregnancy

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It is over a decade since it was first proposed that successful pregnancy is biased towards T helper type 2 (Th2) immunity, while pathological pregnancies, such as pre-eclampsia, are characterized by cell-mediated (Th1) immunity. The Th1/Th2 paradigm however, is too simplistic. In the mouse, production of type 1 cytokines at implantation sites has been found essential for normal implantation. In man, normal pregnancy is associated with a systemic inflammatory response, which is exaggerated in pre-eclampsia. In the past, T helper cells have been considered the mediators of these altered immune responses and lymphocytes of the innate immune system (natural killer (NK) and NKT cells) have been disregarded. Using novel type 1 (interleukin-18 receptor) and type 2 (ST2L) lymphocyte function markers we have shown that compared with non-pregnant women, type 1 cells are depressed in normal pregnancy but not in pre-eclamptic women, confirming previous findings. However, predominant changes are in NK and NKT populations, rather than in Th or T cytotoxic cell populations. These results indicate that immunoregulation in pregnancy could also be a function of the innate, in addition to the adaptive immune system. We propose that the activation of type 1 immunity in pre-eclampsia is due to stimulation of the maternal innate immune system by syncytial debris, rather than activation of maternal T cells by fetal (paternal) antigens.

02-02

Uterine receptivity and the tripod IL-18, IL-15 and IL-12 in humans

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The implantation process, currently thought to be the most critical step in achieving a successful early pregnancy, remains one of the most important unsolved processes for reproductive medicine. It depends on uterine dependent and embryo specific events, which need to be critically coordinated. Early embryo signalling following a maternal hormonal or cytokine mediated preparation phase seem to be involved in stages immediately before, during and just after the apposition step to permit the adequate proliferation of stroma. Our objective is to develop guidelines and diagnostic tools pertinent to appreciate the uterine receptivity. There is an accumulating body of data which strongly suggest that both implantation and uterine receptivity are controlled primarily, though not exclusively, by locally acting growth factors and cytokines, some under steroid control. We previously explore some specific cytokines (IL-12, IL-15 and IL-18) in the endometrium and in the uterine luminal environment that allow to distinct pattern of abnormal uterine receptivity. The identification of these distinct patterns of abnormal uterine receptivity and the possibility that others will be uncovered by further exploration of the local cytokine network strongly suggest that no single therapeutic scheme can correct all cases of implantation failure and should be adapted for each patient.

Short oral presentations

02-03

Pregnancy specific suppression of Th1 immune responses in T-cells is through specific down-regulation of T-bet expression which is regulated by NF- κ B

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Pregnancy is associated with suppression of Th1 immune responses, and bias towards Th2 immune responses. The molecular mechanisms that govern this switch in T-cell reactivity are unknown. We assessed the expression of the Th1 and Th2 cytokine transcription factors T-bet and GATA-3,

respectively. We isolated PBMCs from non-pregnant ($n = 10$) and pregnant females ($n = 10$) and demonstrated that T-bet is down regulated in pregnancy. T-bet was detected at low levels in the nuclear fraction of PBMCs from unstimulated non-pregnant females, but not from pregnant females. In response to PMA/ionomycin the level of T-bet increased in the nuclear fraction of PBMCs from non-pregnant, but not pregnant females. In contrast, we were unable to demonstrate significant levels of GATA-3 expression in the nuclear fraction of either unstimulated or stimulated PBMCs. We have previously shown that NF- κ B is down-regulated in T-cells in pregnancy which results in suppression of the Th1 cytokines IL-2 and IFN γ . We now demonstrate that down regulation of NF- κ B results in diminished expression of T-bet and subsequently Th1 cytokine production. Increasing concentrations of PMA/ionomycin, increased NF- κ B translocation which increased T-bet expression and the production of IFN γ and IL-2. Conversely in the presence of SN50, NF- κ B translocation in response to PMA is suppressed resulting in diminished T-bet expression and subsequently reduced Th1 cytokine production. Thus, maintenance of the cytokine environment that promotes pregnancy success is mediated via strict regulation of the Th1 cytokine transcription factor, T-bet.

02-04

Chemokine and chemokine receptor expression at the fetomaternal interface during pregnancy in normal and abortion-prone mice

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Pregnancy failure is thought to be a process influenced by an increased Th1/Th2 ratio. In this context, the role of cytokines is well analysed, but only little is known about chemokines and their receptors. Thus, the aim of this study was to investigate the expression levels of Th1- and Th2-associated chemokines and chemokine receptors at the fetomaternal interface from mice having normal pregnancy or undergoing spontaneous abortion. We analysed mRNA expression of several molecules (CXCR3,

CCR5, CCR10, MIP-1 α , RANTES, IP-10 and TARC) normalised to β -actin in placental and decidual tissue by employing Real-time-RT-PCR technique. Tissues were taken on day 14 of pregnancy. The mRNA expression of Th2-associated molecules was not altered during abortion, but the expression of the Th1-associated molecules CCR5, MIP-1 α and RANTES was highly up-regulated in deciduas from abortion-prone mice (20% abortion) compared to normal pregnant mice (0% of abortion). These results suggest that CCR5 and its both ligands play a vital role in abortion pathology, probably by allowing the trafficking of Th1 cells into the fetal-maternal interface.

02-05

Progesterone induced blocking factor in normal and growth-restricted ovine placentae

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Progesterone-induced blocking factor (PIBF) was first identified as a 34 kDa immunomodulatory product of pregnancy lymphocytes. Recently, more isoforms and progesterone-independent production were reported. Ovine placental PIBF was investigated in a model of nutrition-restricted placental growth with low circulating maternal progesterone. Ewes were fed a high (H) or moderate (M) intake, inducing restricted vs normal term placental size. Day 50, 80 and 130 placentae ($n = 9, 14, 22$ /treatment/stage respectively) were subjected to PIBF immuno-histochemistry (IHC) and Western blot (WB). Day 130 ewes were categorised into with ($n = 14$) and without fetal IUGR ($n = 8$). PIBF immunolocalised to maternal epithelium and fetal trophoctoderm. At Day 130, PIBF immunostaining correlated with placental size ($P < 0.05$, $R^2 = 0.10$, $n = 43$:M+H). Surprisingly, H group PIBF inversely correlated with progesterone ($P < 0.05$, $R^2 = 0.22$, $n = 22$: no correlation in controls). WB bands of 30, 34, 54 and 90 kDa were identified (similar to the human). Placental PIBF changed significantly across gestation: maximum at day 80 (IHC and 34 kDa WB: $P < 0.05$), co-incident with the apex in

placental growth. Day 50 PIBF was higher ($P < 0.05$) in H vs M placentae (IHC) and the 34 kDa isoform higher at days 50 and 80 but absent in day 130 H animals with IUGR. In conclusion, ovine placental PIBF may be partly progesterone-independent, with the 34 kDa isoform possibly implicated in IUGR.

02-06

Ovine Progesterone-Induced Blocking Factor: identification and regulation during the oestrous cycle and early pregnancy

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Progesterone-induced blocking factor (PIBF) is immunomodulatory and implicated in the maintenance of human and murine pregnancy. However, the precise role and regulation of PIBF in implantation is poorly documented. In the sheep, which has an extended implantation period, we have identified an ovine PIBF ortholog. Changes in uterine PIBF expression and circulating progesterone concentrations during the oestrous cycle and peri-implantation period were investigated. A partial cDNA encoding an endometrial ovine PIBF gene was cloned and then confirmed by DNA sequencing. As in the human, Northern-blot analyses showed a broad tissue distribution of PIBF transcripts. Between days 10 and 17 of the oestrous cycle, when circulating progesterone decreased (10.6 to <1 ng/ml, $n = 5$ /stage), the expression of PIBF transcripts remained stable in the caruncles and inter-caruncular zones. Similarly, between days 12 and 16 of pregnancy ($n = 3$ /stage) levels of PIBF transcripts did not vary and conceptus-derived interferon- τ did not alter PIBF expression *in-vivo*. Although circulating progesterone was unchanged during the first third of pregnancy (~ 5 ng/ml), uterine PIBF gene expression fell ($P < 0.001$). In conclusion, the stability of PIBF during the peri-implantation period suggests that it may be required to establish pregnancy. However, uterine PIBF gene expression does not appear to be only progesterone-dependent in the ewe.

02-07

Maternal immune responses during ovine pregnancy

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Sheep are susceptible to a wide range of pathogens that cause reproductive failure and abortion. The most common cause of infectious ovine abortion in the UK is *Chlamydophila abortus*, a bacterium closely related to *Chlamydia trachomatis*, the most common cause of sexually-transmitted venereal disease in humans. A common feature of chlamydial infections is persistence. This is problematic for implementation of control strategies in sheep, since current serological diagnostic tests do not detect persistently-infected sheep before they abort. Ovine chlamydial abortion follows a very distinct pattern of disease pathogenesis, with infection of the placenta and abortion occurring in the latter stages of pregnancy, even when ewes are infected prior to conception. Host immune control is exerted predominantly through interferon-gamma (IFN- γ). In light of experimental evidence that maternal IFN- γ production is down-modulated in humans and rodents, we hypothesised that a similar down-regulation in sheep could account for recrudescence of a persistent extra-uterine *C. abortus* infection leading to placental invasion and abortion. To test this hypothesis we studied proliferation and IFN- γ production by peripheral blood mononuclear cells (PBMC) from sheep immunised with nominal antigen (Ova). PBMC were collected regularly from sheep throughout pregnancy or from non-pregnant controls and restimulated *in vitro* with Ova. Results showed that there is not suppression of either antigen-driven proliferation or IFN- γ production during pregnancy in sheep.

02-08

Effect of Tumour Necrosis Factor alpha on ovarian folliculogenesis

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Uterine bacterial contamination is ubiquitous in cattle after parturition, causing slower growth of the first postpartum dominant follicle and lower peripheral plasma oestradiol concentrations. Uterine infection is also associated with increased peripheral plasma concentrations of tumor necrosis factor (TNF α). The aim of the present study was to determine the effects of TNF α on ovarian folliculogenesis. Starting twenty-four hours after oestrous, 0.1 μ g TNF α or vehicle was administered intrauterine four times daily for nine days to Holstein heifers, and ovarian folliculogenesis monitored. *In vitro*, cultured bovine granulosa cells from selected or dominant follicles were treated with 0, 1, 10 or 100 ng/ml TNF α and oestradiol (E₂) secretion measured by radioimmunoassay. Animals infused with TNF α took longer to reach follicular dominance (6.3 vs 4.6 days, P = 0.09) and to ovulate (10.0 vs 7.2 days, P = 0.08) with only 4/8 treated animals ovulating compared with 6/7 of controls. Granulosa cells treated with 1, 10 or 100 ng/ml TNF α produced less oestradiol than controls (selected: 1.05, 0.82 and 0.72 vs 1.73 ng/ml E₂ per 10⁶ cells respectively, P < 0.01; dominant: 2.45, 2.09 and 2.38 vs 3.35 ng/ml E₂ per 10⁶ cells respectively). In conclusion, TNF α perturbs folliculogenesis and disrupts ovulation *in vivo*, and suppresses oestradiol production from bovine granulosa cells *in vitro*.

Poster presentations

02-09

Investigation of IL-18 gene promoter polymorphisms in patient with hidatidiform mole

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Background: Gestational trophoblastic tumors (GTTs) include a range of disease of malignant and non malignant features. Epidemiological studies indicate that the incidence of GTTs is more frequent in oriental regions in comparison to western populations. Although in most cases of GTTs, after evacuation, disease recurrence is not seen, the initiation of malignancy after consequent gestation should be expected. IL-18, which is predominantly secreted by activated monocyte/macrophage, is a multifunctional

cytokine involved in the regulation of innate and acquired immune response. In this study the frequency of single nucleotide gene polymorphism at the IL-18 promoter region was investigated in patients with Hidatidiform Mole and healthy control subjects.

Method: DNA from peripheral blood of 70 H.Mole patients and 103 apparently healthy subjects were evaluated for changes in the IL-18 gene promoter (position -137 G/C and position -607 C/A) by AS-PCR (allele specific-PCR) technique.

Results: Results indicated that the frequency of GG, CC and GC at position -137 and CC, AA and CA at position -607 in patients and control were statistically insignificant (P > 0.05).

Conclusion: Results of this investigation conclude that there was no significant difference in the frequency of polymorphism in promoter of IL-18 gene in H.Mole and healthy normal subjects.

02-10

Maternal serum levels of transforming growth factor β 1 in normal and preeclamptic pregnancies

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Successful pregnancy in allopregnant women depends upon the control of graft rejection mechanisms. It has been suggested that immunosuppressive cytokines contribute to successful pregnancy and transplantation. Transforming growth factor beta (TGF- β) exhibits potent immunoregulatory and anti-inflammatory properties which might prolong graft survival. Recent reports suggest a role for TGF- β in the generation of T-regulatory lymphocytes. These regulatory lymphocytes preserve tolerance to peripheral self antigens and may control the response to allogeneic tissues to promote transplantation tolerance. The role of TGF- β in trophoblast differentiation and hypertension prompted us in evaluation of assaying maternal serum TGF- β 1 level in normal allopregnant women and in pregnancies complicated by preeclampsia (PE). PE is a pregnancy complication involving increased blood pressure, proteinuria and end organ damage. Sixty one pregnant preeclamptic women (32 cases with severe and 29 with

mild PE), 22 normotensive healthy pregnant, and 20 non pregnant controls formed these study groups. The active form of TGF- β 1 in serum from all cases was investigated by indirect ELISA technique. The results showed that the TGF- β 1 level is higher in all three pregnant groups compared with the non-preg-

nant controls. No significant changes in serum levels of TGF- β 1 was found in PE compared with normal pregnancy. The results suggest that: (1) TGF- β 1 may function as a regulatory factor in fetal allograft survival during pregnancy, (2) TGF- β 1 does not have a pathophysiological role in PE.

T REGULATORY CELLS AND OTHER FORMS OF IMMUNOREGULATION

Plenary lectures

03-01

The role of regulatory T cells in pregnancy

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Despite the presence of T cells recognising paternal alloantigen the maternal immune system does not reject the fetus. We have demonstrated that regulatory T cells play a crucial role in the protection of the fetus from an attack by the maternal immune system. Indeed, pregnancy induces a systemic expansion of the maternal CD25⁺ T cells capable of suppressing allo-responses. In the absence of regulatory T cells the maternal immune system launches an aggressive response directed against the fetus leading to a failure of gestation due to immunological rejection. Localized mechanisms have been shown to contribute to fetal immune evasion, but they are insufficient to prevent an attack of the fetus in the absence of regulatory T cells. Recently we were able to show that every time a female approaches estrus, regulatory T cells are recruited to and/or retained in the uterus. This suggests that the immune system creates a 'tolerogenic' environment in preparation of a possible implantation prior to fertilisation. This either becomes more pronounced during pregnancy or ebbs away in di-estrus.

03-02

CD4⁺CD25⁺ T regulatory cells mediate tolerance at the fetal-maternal interface by creating a privileged tolerant microenvironment

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The mechanisms underlying immune tolerance during pregnancy are poorly understood. We recently

reported diminished number and function of Treg in abortion-prone mice. Furthermore, the adoptive transfer of CD4⁺CD25⁺ Treg cells from normal pregnant mice could completely prevent fetal rejection. These data confirm that pregnancy-induced Treg cells might play a vital role in maternal tolerance to the allogeneic fetus. We also observed that that vaccination of CBA/J females (H2^k) with splenocytes from BALB/c male (H2^d) previous to pairing with DBA/2J (H2^d), which prevents abortion, expanded the peripheral and thymus Treg subpopulations. Moreover, Treg isolated from these mice rescued from abortion were able to prevent abortion when transferred into DBA/2J-mated CBA/J females. Interestingly, the transfer of Treg generated in third party combinations could not prevent abortion. Our results strongly suggest that Treg act in an antigen-specific manner during pregnancy. Further data from our group suggest that Treg-treatment can not prevent T cell infiltration or high Th1 levels at the fetal-maternal interface but is able to create a privileged tolerant microenvironment, further shedding light onto the molecular mechanisms involved in pregnancy tolerance.

Short oral presentations

03-03

Heme Oxygenase-1 (HO-1) prevents murine abortion

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The placenta can be considered as a naturally occurring allograft, since it expresses both maternal and paternal antigens. Although the physiological pathways protecting the fetus from rejection remain unclear, mechanisms leading to a successful pregnancy are thought to be comparable to those leading to allograft acceptance. HO-1 locally protects against rejection in transplantation models, due to its anti-oxidant, anti-inflammatory and cytoprotective functions. HO-1 is further implied in placental vascular

proliferation and cell growth. Based on previous data on low HO-1 levels in placenta from mice undergoing immune abortion, we hypothesized that an up-regulation of HO-1 during implantation would avoid fetal rejection. Therefore, we treated abortion-prone mice (DBA/2J-mated CBA/J females) with Co-PP or an adenovirus containing HO-1/GFP and included Zn-PP- or Ad-GFP-treated mice as controls. BALB/c-mated CBA/J females served as normal pregnant controls. All treatments were done on day 4–5 of pregnancy, i.e. during implantation. Up-regulation of HO-1 by both, Co-PP or AdHO-1/GFP prevented abortion, while the down-regulation of HO-1 by Zn-PP boosted abortion. In the light of our results, we propose HO-1 as a key regulator of pregnancy success. HO-1 would exert its action at the fetal-maternal interface by up-regulating the Th2/Th1 cytokines ratio and protecting tissues from apoptosis. These data open new therapeutic opportunities for failing pregnancies.

03-04

CD25-positive cells during pregnancy: differential profile of cytokines and soluble MHC antigens produced by CD25⁺CD4⁺ and CD25⁺CD8⁺ cells

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The development of a maternal anti-paternal suppressive activity during pregnancy is at least one of the mechanisms that protect the fetal allograft from immune attack. Many studies in the 80's had demonstrated the presence of specific Ts1 and Ts2 cell populations capable to suppress a mixed lymphocyte reaction against paternal lymphocytes *via* Ia-bearing dimeric soluble factors. Nowadays, the suppressive function is attributed regulatory CD25⁺ T cells. Revisiting the area of immune suppression during pregnancy, the presence study was designated to follow the kinetics of CD25⁺, define the cytokine production profile of CD25⁺CD4⁺ and CD25⁺CD8⁺ cells and examine the production of soluble class I and class II MHC antigens. Following up the kinetics of CD25⁺ cells in the spleen of pregnant mice from day 7 to 14 of gestation by immunofluorescence, maximal percentages of CD25⁺ cell populations were observed on the 12th day of preg-

nancy. The CD25⁺ cell populations were thereafter isolated from the spleen of day 12-pregnant mice, sorted into CD4⁺ and CD8⁺ cells and cultured for 24, 48 or 72 hours. Only the CD25⁺CD4⁺ cell population predominately showed intracellular induction of interleukin-10 (IL-10) and interleukin-3 (IL-3), whereas small amounts of IL-10, IL-3 and interferon- γ (IFN- γ) were detected in the culture supernatants of both CD25⁺CD4⁺ and CD25⁺CD8⁺ cell populations. Interestingly, a statistically significant percentage of CD25⁺CD4⁺ cells contained intracellular class II MHC antigens, whereas a statistically significant percentage of CD25⁺CD8⁺ cells contained intracellular class I MHC antigens. Such distinction was detected in the culture supernatants of these cells. These results indicate that the CD25⁺ cell populations could indeed represent the suppressive T cell population during pregnancy which would mediate their effect through specifically produced soluble MHC antigens.

Poster presentations

03-05

Analysis of endometrial myeloid and lymphoid dendritic cells during mouse estrous cycle

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Immune cells including macrophages, MHC-II positive cells and T lymphocytes have been reported in the uterus of a number of species including mice, rats and human. However no study so far has been performed considering the presence of dendritic cells (DC) in mouse uterus. In this study the stage of the estrous cycle was determined by examination of vaginal smears. Uteri of mice were removed in each stage of estrous cycle. Frozen sections 5 μ m thick were cut on a cryostat and stained with antibodies against CD11c, CD11b, CD8 α , CD86, CD205 and MHCII. Our results showed that DCs were present throughout the estrous cycle in mice endometrium, but their frequency was highest in estrous and lowest in proestrous. There was no statistical difference between different stages of estrous cycle in lymphoid (CD11c⁺,CD8 α ⁺) / myeloid (CD11c⁺, CD11b⁺) DC ratio, although this ratio was higher in

estrous phase. Indeed DCs were present in large numbers around the glandular epithelium at estrous, relative to those seen at diestrous. Nearly all of endometrial DCs expressed CD86 and MHCII. This study demonstrated that DCs are present in mouse endometrium. Considering the capability of DCs in inducing immunity as well as tolerance, their presence in the endometrium might have a critical role in the regulation of maternal immune responses to paternal antigens during insemination and pregnancy.

03-06

Characterization of decidual dendritic cells during mouse pregnancy

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Dendritic Cells (DC) are professional antigen presenting cells capable of induction of primary immune responses as well as immunologic tolerance. Myeloid (MDC) and lymphoid (LDC) subsets of murine DCs are able to shift cytokine responses of T cells toward Th2 and Th1 profile, respectively. So, DCs would be suitable candidates that mediate the balance of maternal immune responses to conception. We analyzed local (uterus) and systemic (spleen) DCs in a murine model. C57BL/6 mated Balb/c female mice with vaginal plugs were killed at early, middle and late pregnancy. Frozen sections of uterus and spleen at each stage of pregnancy were stained with CD11c and MHC-II. Also two-color immunohistochemistry was carried out using anti CD11c and one of the antibodies against CD11b, CD8 α , CD86, and CD205. Using morphometric analysis the average density of DCs and relative percentage of myeloid (CD11c⁺, CD11b⁺) and lymphoid DCs (CD11c⁺, CD8⁺) were determined at each stage. Our results showed that DCs were present throughout the pregnancy in decidua. The average density of DCs was significantly higher at

early compared to middle or late stages of pregnancy. The LDC:MDC ratio was significantly higher at mid gestation compared with other stages. Although total number of splenic DCs decreased; the LDC:MDC ratio was significantly elevated in mid gestation. Nearly all decidual and splenic DCs expressed CD86 and MHC-II. In conclusion it seems that the balance of MDC and LDC is finely tuned throughout pregnancy, pointing an eminent immunoregulatory role of DCs in maintenance of pregnancy.

03-07

Pregnancy-protective Treg are antigen-specific

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During pregnancy the immunological system tolerates foreign (paternal) alloantigens. To explain this paradoxical situation several hypothesis have been proposed. Recently, CD4⁺CD25⁺ regulatory T cells (Tregs) were claimed to play an important role in preventing the rejection of the semiallogeneic fetus by suppressing allo-specific Th1 responses. Since previous data from our laboratory suggested that Treg may be previously activated at the fetal-maternal interface after encountering alloantigens, the aim of the present study was to investigate whether Treg cells act in an antigen-specific manner. CBA/J females were therefore mated with CBA/J, C57/BL6 or Balb/c mice and on the day 14 of pregnancy, the number and phenotype of Treg cells was analysed. Further, Tregs isolated from 14-days pregnant mice (from all these combinations) were adoptively transferred into DBA/2J-mated CBA/J female undergoing abortion. Interestingly, Treg cells of the CBA/J x BALB/c or CBA/J x CBA/J combinations but not those from the CBA/J x C57/BL6 combination could prevent abortion. These results suggest that Treg cells need to be primed *in vivo* by allo- or synantigens for being effective *in vivo*.

03-08

Flow cytometric analysis of endometrial lymphocytes during implantation and early pig pregnancy

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The involvement of immune cells in materno-fetal relationship during implantation and early pregnancy in epitheliochorial placenta is not well established. The endometrial CD45⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD16⁺ and $\gamma\delta$ TCR⁺ lymphocytes of 10, 15, 20, 30 and 40 days pregnant sows were analyzed by flow cytometry. Cells were isolated by mechanical dispersal technique from endometrial tissue at and between attachment sites. It was found that the suppressor/cytotoxic (CD3⁺CD8⁺) lymphocytes were dominant in the pig endometrium (60–85%) during early pregnancy with peak in the initial phase of the implantation (15th day). The second abundant endometrial lymphocyte population was that of uNK cells (CD16⁺) - 16–42% showing significant decrease during early pregnancy. For the first time the presence of endometrial $\gamma\delta$ TCR⁺ cells in pig pregnancy it was determined. They were in relatively small percentage between 15th and 30th day (5–10%) and significantly increased on 40th day (23%). No significant difference in CD3⁺4⁺, CD3⁺8⁺, CD3⁻8⁺, CD16⁺ and $\gamma\delta$ TCR⁺ cells at attachment and between attachment sites was observed. In conclusion, pregnancy induces dynamic changes in the endometrial lymphocytes of pigs. The numbers of CD3⁺CD8⁺, CD3⁺CD4⁺, CD16⁺ and $\gamma\delta$ TCR⁺ cells as well as the intensity of CD45 cell surface molecule expression were affected by stage of gestation but not by proximity of conceptuses.

03-09

Soluble class II antigens isolated from maternal serum during pregnancy specifically modulate the immune response

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Secreted forms of MHC class II molecules have been detected in serum and nearly all human body fluids, such as saliva, sweat, cerebrospinal fluids and urine of healthy and diseased individuals. Their role during pregnancy is of great importance, since there is direct relevance between their concentration and maternal immunostimulation. Since the immunomodulatory effect of secreted MHC class II molecules has not yet been defined, through a series of *in vitro* experiments, we tried to clarify the profile of s-MHC class II molecules (SCII). Serum was taken from mice during their 9th and 11th day of pregnancy and class II molecules were isolated through a dual system of chromatography columns, first an affinity protein A column and then an immunoaffinity column, where Sepharose 6MB was coupled with HB3, a monoclonal antibody for class II molecules. The fractions were examined for protein activity through ELISA experiments and analysed by SDS PAGE, where 3 bands of MW = 62 kDa, 59 kDa and 56 kDa were detected. These mixtures of isolated class II molecules were applied to functional *in vitro* experiments to test their involvement in spleen cell proliferation, cytokine production and alteration of the T/B/Mac cell ratio – our results demonstrate that SCII molecules induce spleen cell proliferation and in particular SCIID9 (SCII from day 9 of pregnancy) molecules induce macrophage and B cell proliferation, while SCIID11 molecules induce T cell proliferation. Moreover SCII molecules induce production of Th2 type cytokines, while they suppress the secretion of Th1 type cytokines.

INDOLEAMINE 2,3-DIOXYGENASE AND MATERNO-FETAL INTERACTIONS

Plenary lectures

04-01

Indoleamine 2,3-dioxygenase in materno-fetal interactions

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Cells expressing indoleamine 2,3-dioxygenase (IDO) – e. g. antigen presenting cells induced by regulatory T cells by CTLA-4 binding to B7 molecules - deplete their vicinity of tryptophan and induce accumulation of tryptophan metabolites. This inhibits T cell proliferation by activating the GCN2 kinase pathway, inducing a cell cycle arrest in the G1 phase. IDO has been shown to be crucial for successful pregnancy in the mouse. Blocking of IDO leads to rejection of allogeneic but not syngeneic fetuses which is provoked by MHC-I difference and depends on the activity of maternal T cells. The consequences are deposition of complement at the materno-fetal interface, T cell infiltration, inflammation, hemorrhagic necrosis, and eventually embryo resorption. However, this issue has raised a puzzle recently as it is not well understood why allogeneic matings in which both parents were IDO-deficient produce offspring at normal size and rate. Several studies have given quite divergent results for the expression pattern of IDO at the feto-maternal interface. It is questionable, though, whether IDO acts locally or whether tolerance induction by IDO takes rather place in regional lymph nodes.

04-02

Implications of indoleamine 2, 3-dioxygenase in the human placenta

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The enzyme indoleamine 2, 3-dioxygenase (IDO) has been the focus of great interest over the last seven years because of the discovery of its role in

regulation of T lymphocyte function. By utilization as a substrate (and hence localized depletion) of the amino acid L-tryptophan, cells expressing IDO are able to produce a microenvironment around them in which there is profound T cell suppression. The function of this enzyme producing such 'immunosuppression by starvation' can be explored by two classical enzymological manoeuvres: either by inhibition of the enzyme's active site using a competitive inhibitor (such as 1-methyl D, L-tryptophan), or by over-riding of the effect of the enzyme's activity by addition of excess substrate (tryptophan supplementation). Both of these have been used to examine IDO biology in distinct areas of immunobiology (for example in exploring how tumour cells avoid immune attack, for example in studying how specific populations of dendritic cells regulate the immune response). Much attention, following seminal findings in the pregnant mouse by EA Munn and A Mellor, has additionally recently been focussed on the role of IDO in materno-fetal interactions. I will discuss, in the context of human placental biology, the evidence for a role of IDO in pregnancy in health and disease; and will focus in particular on other implications (for example regarding L-tryptophan transport) that will need to be further explored before its integrated role in human reproductive immunology can be fully addressed.

Short oral presentations

04-03

Phagocytosis of shed trophoblasts increases the expression of indoleamine 2,3-dioxygenase by U937 cells

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Introduction: The fetus and placenta are immunologically foreign to the mother, yet large quantities of trophoblasts are shed daily from the placenta into the maternal blood. A mechanism must exist to clear the trophoblasts from the maternal circulation but this mechanism is unknown. It is likely that macr-

ophages are important in clearing shed trophoblasts but phagocytosis of foreign cells by macrophages usually leads to an immune response. We undertook this study to investigate the effect on macrophages of phagocytosing shed trophoblasts.

Methods: We induced U937 cells to an adhesive, macrophage-like phenotype by treatment with PMA. We labelled villous explants with green fluorescent cell tracker stain then harvested trophoblasts shed from explant cultures and exposed them to the U937 cells. Phagocytosis was examined by confocal microscopy and the expression of indoleamine 2,3 dioxygenase (IDO) by the U937 cells was examined by ELISA.

Results: After exposure of the shed trophoblasts to U937 cells green fluorescent trophoblast-derived particles were present within the U937 cells. The expression of IDO by the U937 cells more than doubled ($p < 0.01$) following phagocytosis of the shed trophoblasts.

Conclusions: Phagocytosis of shed (apoptotic) trophoblasts leads to an immunosuppressive type of response by macrophages. Thus, trophoblast shedding may be important for the development of maternal immune tolerance towards placental/fetal antigens.

04-04

Gene expression and signaling pathway in trophoblast cells by IFN- γ

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Interferon (IFN)- γ is considered an abortion-inducing factor even though this cytokine has been localized at the maternal-fetal interface in the first half of the gestation during normal pregnancy in mice and other species. Experimental evidences also point to specific functions of this cytokine in stimulating proliferation and phagocytosis and, inducing indoleamine 2,3-dioxygenase (IDO) protection mechanisms in trophoblast cells. To better understanding the behavior of IFN- γ -treated trophoblast cells, in this study, special attention has been done to the induced-gene expression of mice ectoplacental

cones obtained at the day 7.5 of gestation and treated with this cytokine at the physiological dose of 100 U/mL. The presence of IFN- γ strongly modified the expression of 42 genes when determined through semi quantitative RT-PCR and DNA macro-arrangements. Among these genes, particularly interesting were those related to IFN- γ -induced transcription factors such as IRF-1 and SGF3 and, the expression of genes that encode the main proteins of the IFN- γ -signaling pathway, which include JAK1, JAK2 and STAT1. Phosphorilated STAT 1 protein at the trophoblast nuclei was also dependent of IFN- γ induction. Since this cytokine is able to induce a specific gene response, unrelated to cell death it deserves to be deeper studied on the framework of the roles played by the placenta for adaptation to the maternal environment and immunological adaptations of pregnancy.

04-05

Bimodal effect of nitric oxide in the indoleamine-2,3-dioxygenase activity: Implications in maternal tolerance

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Indoleamine-2,3-dioxygenase (IDO) is the first limiting enzyme of tryptophan degradation, and by this mechanism provokes T-cell suppression. Placenta expresses IDO, which participates in preventing maternal immune rejection of the foetus. Both infiltrating antigen presenting cells and trophoblast express constitutive and inducible nitric oxide synthases (NOS). Low NO production by constitutive NOS is usually produced for maintaining the normal vascular tone, while high NO output produced by inducible NOS is found in an inflammatory process. In this work we studied the effect of these NOS in IDO activity. We selected a model of monocytic cell line U937 that express IDO and both constitutive and inducible NOS. NO at high micromolar concentrations blocked IDO activity, while at low micromolar concentrations NO could stimulate IDO activity ($p < 0.05$). The NOS inhibitors L-NMMA and aminoguanidine also decreased IDO activity ($p < 0.01$). This effect was related to enzyme activity and not to IDO protein synthesis, as demonstrated by western blot. The calmodulin inhibitor

W7, that blocked constitutive NOS, also inhibited IDO activity ($p < 0.01$). The effect of NOS in IDO activity was not through GMPc production. Besides, immunoprecipitation showed that there is a nitration of the IDO protein. In conclusion, these results suggest that NO at low concentrations, could favour a tolerating action by inducing IDO activity, while at high concentrations it could participate in rejection.

04-06

Indoleamine 2,3 dioxygenase- and tryptophan-dependent regulation of HLA-G cell-surface expression

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Nonclassical human leukocyte antigen (HLA) class I molecule HLA-G and indoleamine 2,3 dioxygenase (INDO) have been shown to play crucial immuno-suppressive roles in fetal-maternal tolerance in humans and mice, respectively. HLA-G inhibits NK and T cell function by high-affinity interaction with inhibitory receptors, and INDO acts by depleting the surrounding microenvironment of the essential amino acid tryptophan, thus inhibiting T cell proliferation. We investigated whether HLA-G expression and INDO function were linked. Working with antigen-presenting cell (APC) lines, monocytes, and dendritic cells, we found that functional inhibition of INDO by 1-methyl-tryptophan (IMT) induced HLA-G1 cell-surface expression by APCs that were originally cell-surface negative. On the contrary, functional boost of INDO by high tryptophan concentrations induced a complete loss of HLA-G1 cell surface expression by APCs lines and monocytes that were originally cell-surface HLA-G1-positive. Interestingly, IMT and tryptophan both induced HLA-G cell-surface expression in DC whose stimulatory function was abrogated. This mechanism of cell-surface HLA-G

regulation was posttranslational since HLA-G protein cell contents were unaffected by the treatments. Finally, HLA-G cell surface expression regulation by INDO relates to INDO function, but not to tryptophan catabolism.

04-07

A critical role for galectin-1 in the maintenance of maternal-fetal tolerance

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Introduction: Galectin (gal)-1, one of the earliest-identified members of a family of lectins, occurs in a variety of tissues undergoing dramatic changes, e.g. the fetoplacental unit. An intriguing function of gal-1 is its modulation of immune responses. Thus, gal-1 is likely key modulators of pregnancy maintenance.

Materials and methods: Uterine gal-1 expression was evaluated in uterine tissue from normal, failing and progesterone-rescued murine pregnancies by immunohistochemistry. Additionally, mice with failing pregnancies triggered by stress exposure were treated with recombinant gal-1. Phenotype and maturity of dendritic cells (DC) as well as tolerance mechanisms at the fetomaternal interface were also evaluated in the respective groups by flow cytometry and ELISA. The immunomodulatory effects of DC on allogeneic mixed-lymphocyte responses upon gal-1 treatment were also tested *in vitro*.

Results: Uterine expression of gal-1 is down-regulated in failing pregnancies and restored by progesterone. Treatment with gal-1 prevents fetal rejection by increasing the presence of uterine gal-1^{bright} DC population. Subsequently, gal-1^{bright} DC mediate fetal tolerance via a Th2 skew, prevention of Th1 cells, up-regulation of indoleamine 2, 3-dioxygenase and CD4⁺CD25^{bright} T regulatory cells.

Conclusions: Our data provide clear evidence for the modulatory function of gal-1 in pregnancy maintenance and suggest gal-1 as a promising therapeutic novel agent to improve reproductive outcome.

GAMETIC IMMUNOLOGY

Plenary lectures

05-01

The multi-tasking of complement regulatory proteins [CRPs] on sperm?PM Johnson¹, JA Cummerson¹, BF Flanagan¹, LE Clift¹, K Dvorakova²¹Division of Immunology, University of Liverpool, UK, and ²Department of Development Biology, Charles University, Prague, Czech Republic

Membrane cofactor protein [MCP; CD46] is a CRP expressed widely on human cells but, by sperm, solely as an unusual molecular isoform on the inner acrosomal membrane [IAM] which becomes surface exposed only after the acrosome reaction [AR]. Spermatozoal CD55 and CD59 are considered to be predominantly localised on the plasma membrane, although we have demonstrated CD55 is also markedly expressed on the IAM. Antibodies against CD46, but not CD55 or CD59, inhibit binding and penetration of human sperm to zona-free eggs. Antibodies specific for the first short consensus repeat [SCR] domain are most effective. New World monkeys express a CD46 isoform lacking SCR1 on all cells except the IAM of spermatozoa, supporting a role for the SCR1 ectodomain in fertilisation. Also, in rodents, CD46 is not a systemic complement regulator but instead is solely expressed on spermatozoal IAM and no other cells. PCR and immunocytochemistry have indicated that outbred wood mice may fail to express spermatozoal CD46 and, like CD46 knock-out mice, are fertile and undergo an accelerated spontaneous AR. Proposed complement-dependent and -independent roles for CD46 on AR-sperm include protection from local complement activation, involvement in sperm-egg binding/fusion, acrosome stabilisation and opsonisation of redundant sperm.

05-02

Microdomains, diffusion barriers and lipid rafts in sperm cell plasma membranes

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We are using fertilization as a model system for studying how mammalian cells recognise, bind and eventually fuse with each other. Because they are transcriptionally inactive, spermatozoa have a limited repertoire of responses to external signals yet they still have to regulate their fertilizing capacity until they are within the vicinity of the egg. One mechanism for achieving this is to re-position egg-binding molecules from areas of the cell where they are inactive to areas where they are fully active and correctly presented. In some cases this involves active migration across putative diffusion barriers and against large concentration gradients. Using monoclonal antibody probes, high resolution imaging and fluorescence microscopy techniques (AFM, FRAP, FLIP, SPFI and SICM) we have investigated the distribution and diffusion of lipids and proteins on the surface membrane. Sperm membranes are compartmentalised into 6 microdomains each with its own specific complement of antigens. D values for lipid reporter probes differ between microdomains yet lipids are able to diffuse unimpeded across putative diffusion barriers. Complexes of many hundreds of molecules on the other hand, cannot exchange between microdomains suggesting the presence of a 'molecular filter' at domain boundaries. Significantly, a reduction in membrane cholesterol activates signalling cascades within the sperm cell leading to phosphorylation of specific proteins within the acrosomal vesicle. Concomitantly, so-called lipid rafts enlarge and change their surface distribution. Single molecule tracking studies are currently in progress to investigate the presence (or not) of nanometer-sized domains within the membrane and how they respond to external agonists.

Short oral presentations

05-03

Sperm proteome; a source of new functional entities

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Intensive ongoing studies are focused on specific cell type proteome. Novel findings can be important not

only due to the immunological features of the detected epitopes but more to the general cell function. Specifically, spermatozoa have been difficult targets for immune response escaping in most cases to reveal their private-specificities. Sperm proteome approach with variety of antisera should be helpful to delineate novel spermatozoa targets. In our studies several panels of antisperm antibodies have been applied, i.e., a) antibodies raised in a hu-SCID system, b) naturally developed circulating antisperm antibodies (ASA) of infertile males and females, c) from pre-pubertal boys, d) obtained locally from cervical mucus or seminal plasma. 2-D electrophoresis has been then applied and the selected entities cored from the gel and dissected through mass spectrometry. ASA-positive sera samples of infertile males allowed identification of 17 antigens. Much less has been a case concerning analyzed sera samples of infertile women. ASA from seminal plasma and male sera sample showed reactivity to the panel of antigens connected with glycolytic enzymes with specific germ cells isoforms. Interestingly sera from pre-pubertal boys revealed reactions with sperm antigens so far detected on mature sperm. In conclusion: valuable data have been obtained concerning both immunogenicity of detected sperm entities as well as their putative function. Several discovered novel sperm moieties present an exciting opportunity to have a further insight into unknown sperm-specific genes and proteins.

05-04

Characterization of immune response in mice to plasmid DNA encoding human Sperm associated antigen 9 (SPAG9)

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Earlier we reported human sperm associated antigen 9 having structural homology with JNK binding protein is involved in sperm egg interaction (Jagadish et al. (2005) Characterization of a novel human sperm associated antigen 9 (SPAG9) having structural homology with c-Jun NH₂-terminal kinase interacting protein. *Biochem J* 389, 73–82). SPAG9, a single copy gene mapped to the human chromosome 17q21.33, was earlier shown to be expressed

exclusively in testis (Jagadish et al., 2005). Present investigation was undertaken to examine the immunogenicity employing DNA encoding human sperm associated antigen 9 (hSPAG9). The cDNA corresponding to hSPAG9 was cloned in pcDNA3.1 down stream of cytomegalovirus promoter. Immunization of female BALB/cJ mice with pcDNA-hSPAG9 plasmid DNA in saline by intramuscular (i.m.), by adsorbing onto gold microcarriers (delivered by gene gun) generated antibody response against native SPAG9 protein. A dominant IgG1 isotype response was observed in mice immunized with pcDNA-hSPAG9 plasmid DNA delivered by gene gun as compare to a mixed IgG1-IgG2a isotype response in mice immunized with pcDNA-hSPAG9 plasmid DNA delivered by i.m. Further, antibodies generated by pcDNA-hSPAG9 plasmid DNA localized acrosomal compartment of human sperm, inhibited sperm binding/attachment in zona free hamster egg penetration test. These studies for the first time, demonstrate the feasibility of generating an immune response to sperm specific hSPAG9 protein by DNA vaccine and that antibodies thus generated recognize native SPAG9 in human sperm.

Poster presentations

05-05

The influence of eco-toxicological factors on mammalian sperm *in vitro*

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Increasing infertility, due to pathological changes on sperm, has become a serious issue in human population. In order for sperm to gain the fertilizing ability, they must undergo the capacitation, which includes tyrosine phosphorylation and actin polymerization, which is ATP binding dependent. Fluorides in presence of aluminium ions form metal-fluoride complexes, which are analogues of phosphate groups, and can imitate the role of ATP or GTP. We aimed to reveal, whether aluminium-fluoride complexes influence the ability of spermatozoa to capacitate, focusing on actin polymerization and tyrosine phosphorylation. Mouse epididymal sperm were left to capacitate for 3 h, however samples were taken every 1/2 h for evaluation of motility, viability, and

the status of the acrosome vesicle. The organization of F-actin and level of tyrosine phosphorylation were assessed by immunofluorescence assay. Our results indicate a time delayed capacitation process. Aluminium-fluoride-treated-sperm showed a 90-minute-slow-down of actin polymerization in the head acrosomal region. The percentage of ongoing tyrosine phosphorylation was in correlation with results of actin. At the time of 3 h, there was 30% less sperm, which were able to undergo the Ca^{2+} -ionophore induced acrosome reaction, than in a control group. In conclusion, the enhancing overload caused by aluminum-fluorides represents a potential factor, which can have an impact on the structure and function of sperm, hence contributing to a growing infertility in the human population.

05-06

New monoclonal antibody against carp sperm creatine kinase

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Creatine kinase (EC 2.7.3.2) enzymes catalyze reversible transfer of the high-energy N-phosphoryl group from phosphocreatine to ADP. This way of ATP regeneration plays an important role in tissues with intermittently high, fluctuating energy demands, e.g. in skeletal and cardiac muscle, neurons and spermatozoa. In terms of spermatozoa the utilization of high-energy phosphate transport via phosphocreatine shuttle differs with respect to individual species. The newly prepared monoclonal antibody against carp sperm proteins was proved to specifically recognize a protein belonging to the creatine kinase family of carp spermatozoa. Positive reactions were gained in Western blot analysis having used urea, SDS and Triton X-100 extracts. A protein of 42 kDa was labeled. The same types of extracts of human, boar and sturgeon spermatozoa gave no positive reaction. In indirect immunofluorescence experiments slight differences in labeling were observed according to the type of permeabilization (Triton X-100, acetone). No labeling was observed on human, boar and sturgeon spermatozoa under the same conditions. The newly described antibody against the sperm creatine kinase is specific

to carp spermatozoa and is applicable to a large scale of immunochemical techniques. This work was supported by the grant No: 524/03/0178 from the Grant Agency of the Czech Republic.

05-07

Antibodies against glutathione s-transferase mu 5 and lactate dehydrogenase C4 purified from serum of patients with immune infertility impair mouse sperm function *in vitro*

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Human antisperm antibodies (ASA) binding to fertility-related antigens may negatively influence the sperm function which cause immune infertility. Using proteomics approach we identified two mouse surface antigens that cross-reacted with ASA from infertile patients: glutathione s-transferase mu 5 (GSTmu5) and lactate dehydrogenase C4 (LDHC4). In this study we investigated the role of LDHC4 and GSTmu5 for mouse sperm function by antibody mediated inactivation of these proteins *in vitro*. The mouse sperm were incubated with different dilutions of antibodies against LDHC4 or GST-mu5 previously purified from patients serum containing ASA by using of affinity columns. Motility of the mouse sperm was measured by the aid of a CASA system. The effect of the antibodies on acrosome exocytosis in response to calcium ionophore was evaluated by lectin staining. The sperm capacitation was studied by the chlortetracycline test and tyrosine phosphorylation status of sperm proteins. The experiments showed that LDHC4 antibody binding to the sperm reduced significant sperm motility as compared to the controls. The incubation with GSTmu5 antibodies had no effect on sperm motility. The percentage of mouse sperm cells undergoing acrosomal exocytosis was significantly decreased. The percent of cells which acquired the pattern B chlortetracycline fluorescence was lower and depended on the antibody concentration of both LDHC4 and GSTmu5. Block of capacitation was demonstrated at the level of tyrosine phosphorylation of various proteins. These studies give insights into the mechanisms by which ASA might impair sperm function.

05-08

Association of MHC class I antigens with spermatogenesis abnormalities in male patients of *in vitro* fertilization

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VA Bakharev, LN Kuzmichev

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The human major histocompatibility complex (MHC) is being scrutinized by scientists for the links with pre-disposition to different pathologic conditions. The evaluation of MHC class I antigens can be one of the possible paths in determining individual predispositions to certain diseases. We have evaluated three groups of ethnically Russian male patients of IVF. The groups were constituted as follows: 1) with normozoospermia (106 patients), 2) with oligozoospermia, asthenozoospermia, or a combination of those sperm disorders (102 patients) and 3) with teratozoospermia (isolated, or associated with oligozoospermia and/or asthenozoospermia) (109 patients). Positive association of MHC class I antigens with a certain sperm disorder was found only in patients with reduced levels of normally shaped sperm (Group 3) ($P < 0,05$). The risk of developing teratozoospermia >4 was linked to haplotypes A10B35 (4,5) and A26B18 (4,7). The association of both (B18 and B35) antigens increases that risk to 11. MHC class I antigens represent possible immunologic markers of teratozoospermia. A further study of these antigens in patients with abnormalities of spermatogenesis is needed, and it will possibly be relevant in predicting the state of their progeny's health.

05-09

Efficacy of RIBI adjuvant for GnRH vaccines in post-pubescent rats

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Vaccines which stimulate the production of neutralising antibodies against gonadotropin-releasing hormone (GnRH) have application in immunosterilization, immunocontraception, prevention of sexual maturation, and as therapeutic agents for GnRH-dependent diseases including tumors. In a comparative study of GnRH vaccine constructs, post-pubescent Wistar rats (8 weeks old, $n = 7/\text{group}$) were immunized with various vaccine formulations in RIBI adjuvant. Following priming, the animals were boosted at weeks 3 and 7. Vaccine candidates used were: GnRH conjugated to mycobacterial heat shock protein 70 (GnRH-hsp70); GnRH conjugated to keyhole limpet hemocyanin (GnRH-KLH); retroinverso-GnRH; retroinverso-GnRH-hsp70; tandem GnRH; tandem GnRH-hsp70; tandem GnRH-KLH; and a GnRH multiple antigenic peptide incorporating a helper T-cell peptide. The animals were bled at week 9 to measure antibodies and sacrificed at week 14 for testosterone and tissue analyses. The antibody levels correlated with circulating testosterone, testis weight, and size of the urogenital complex. The highest antibody titres were generated by GnRH-KLH, the MAP-Th-GnRH and the tandem GnRH-KLH. Thus, in post-pubescent rats, these three GnRH constructs with the mild adjuvant RIBI functioned as effective vaccines for the reduction of circulating testosterone.

05-10

Association of MHC class I antigens with spermatogenesis abnormalities in male patients of *in vitro* fertilization

SA Diyakonov, Zhi Glinkina, IG Dzenis,
VA Bakharev, LN Kuzmichev

The Scientific Centre for Obstetrics, Gynaecology and Perinatology
RAMS, Moscow, Russia

The human major histocompatibility complex (MHC) is being scrutinized by scientists for the links with pre-disposition to different pathologic conditions. The evaluation of MHC class I antigens can be one of the possible paths in determining individual predispositions to certain diseases. We have evaluated three groups of ethnically Russian male patients of IVF. The groups were constituted as follows: 1) with normozoospermia (106 patients), 2) with oligozoospermia, asthenozoospermia, or a combination of those sperm disorders (102 patients) and 3) with ter-

atozoospermia (isolated, or associated with oligozoospermia and/or asthenozoospermia) (109 patients). Positive association of MHC class I antigens with a certain sperm disorder was found only in patients with reduced levels of normally shaped sperm (Group 3) ($P < 0,05$). The risk of developing teratozoospermia >4 was linked to haplotypes A10B35

(4,5) and A26B18 (4,7). The association of both (B18 and B35) antigens increases that risk to 11. MHC class I antigens represent possible immunologic markers of teratozoospermia. A further study of these antigens in patients with abnormalities of spermatogenesis is needed, and it will possibly be relevant in predicting the state of their progeny's health.

INFERTILITY AND RECURRENT PREGNANCY LOSS

Plenary lectures

06-01

Diagnosis of immune-mediated recurrent spontaneous abortion: an endless quest

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Immune-mediated Recurrent Spontaneous Abortions (RSA) are divided in two major categories: Autoimmune abortions, in which maternal autoimmune reactions affect the development of the placenta and the embryo, and the so-called alloimmune abortions, in which maternal reactions result in the "rejection" of the semiallogeneic embryo. Autoimmune abortions represent one of the clinical manifestations of the Reproductive Autoimmune Syndrome (RAS). Initially, their laboratory diagnosis was based on the detection of lupus anticoagulant and increased levels of anti-cardiolipin antibodies in women's serum. Today, it is recommended to additionally test for antibodies against a panel of phospholipids and plasma proteins binding phospholipids, as well as for other non specific or organ specific autoantibodies (ANA, anti-TPO). Definition of the type, the isotype and the title of antibodies provide information on the underlying autoimmune mechanism/s and are useful in the prognosis of the outcome of current or next pregnancies and the choice of treatment.

Alloimmune abortions and their diagnosis appear much more complicated. To approach them, one has to consider various different immunological mechanisms suggested to get involved in normal pregnancy, and to look for relevant disturbances that could cause damage to the trophoblast. Possible disturbances include: Insufficient recognition of trophoblastic antigens by maternal decidual cells, predominance of a Th1-type maternal response at the fetomaternal interface, increased toxicity of Natural Killer (NK) cells. In practice their diagnostic approach remains difficult. The diagnostic/prognostic value of initially used tests, such as HLA sharing between partners and the absence of "anti-paternal" antibodies in maternal serum (indirect markers of

insufficient recognition of trophoblastic antigens), is currently doubted. On the other hand, the existing question whether peripheral findings reflect reactions at the fetomaternal interface makes it difficult to prove a Th1 shift in aborters. Practically, the estimation of the toxic potential of NK cells by studying their presence on decidua and measuring their peripheral blood subsets and their lytic activity, are the only commonly used tests, which -if correctly interpreted- provide useful information on the underlying pathology.

For the work-up in alloimmune/unexplained abortions, we currently suggest two additional approaches: a) Estimation of epitope matching between maternal inhibitory NK receptors (KIR) and fetal HLA-C allotypes, since it seems that lack of such a matching may predispose to miscarriage because of the absence of signals to inhibit NK cell anti-trophoblast activity. b) Detection of CD3⁺γδ⁺γ9δ2⁺ PB T cells, which -if increased- appear to be a marker of Th1 triggering by infectious agents.

06-02

Stress and pregnancy maintenance: from basic science to the clinic

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Spontaneous abortion is the most common adverse pregnancy outcome, and stress has been suggested to be abortogenic in mice and humans. Stress-and/or pregnancy-related hormones (corticotropin releasing hormone, adrenocorticotropin, and progesterone) might interact with peripheral and local immunocompetent cells, such as certain T cell, mast cells, dendritic cells or NK cells, and result in changes of cytokine production. In an established murine model, abortions can be triggered by exposing the mice to stress during early gestation. Recent data from this model indicated that increased levels of abortogenic Th1 cytokines, a decrease of progesterone and thus, PIBP were attributable for reduced fetal tolerance and hence, incongruous

with successful pregnancy maintenance. Interestingly, data from a prospective study on human pregnancy revealed that women with a clinically normally progressing pregnancy but low levels of progesterone during the first trimester eventually suffered from a miscarriage. These data indicate that stress may lead to increase abortions by altering the endocrine system, which triggers an immune bias towards an abortogenic cytokine profile. Progesterone may be a good marker to identify a putative thread of a miscarriage in human and progesterone replacement therapy may mechanisms of fetal tolerance.

Short oral presentations

06-03

IgG, IgA and IgM antibodies against FSH: serological markers of pathogenic autoimmunity or of normal immunoregulation?

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Introduction: Autoimmune mechanisms are often involved in causing infertility. Among the possible targets of autoantibodies, the follicle-stimulating hormone which regulates the follicular maturation in human ovary is a promising candidate. We aimed to study whether anti-FSH-antibodies might be involved in different clinical types of infertility.

Method of study: The study group consisted of 178 patients (75 with polycystic ovary syndrome, 103 with endometriosis) and 75 pregnant women. Female blood donors formed the control group (n = 85). Indirect ELISA tests were performed using purified FSH as antigen and a synthetic peptide corresponding to the 78-93 region (V14D) of the human FSH β -chain.

Results and conclusion: We showed that anti-FSH-antibodies were present in controls and in pregnant women and their production decreased during pregnancy. Endometriosis and polycystic ovary syndrome were associated with higher values of anti-FSH-IgA, anti-V14D-IgA, and endometriosis with anti-V14D-

IgG. Our data suggest that naturally occurring anti-FSH-IgA could be a marker for infertility in ovarian disorders when presented in higher values.

06-04

EMILIN1 is involved in human trophoblast invasion towards decidual vessels

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The detection of EMILIN1, a connective tissue glycoprotein associated with elastic fibers, at the level of the ectoplacental cone and trophoblast giant cells of developing mouse embryos favoured the idea of a structural as well as of a functional role of this protein in the process of placentation. During the human placenta establishment, a highly migratory subpopulation of extravillous trophoblasts (EVT), originating from anchoring chorionic villi, penetrate and invade the uterine wall and interstitium. In this study we show that, among the several components of the first trimester decidua extracellular matrix, EMILIN1, produced by decidual stromal and smooth muscle uterine cells, is very relevant for EVT migration. EMILIN1 is expressed as a gradient of increasing concentration in the perivascular region of modified vessels. This particular protein distribution is consistent with the haptotactic directional migration observed *in vitro* functional studies displayed by freshly isolated EVT and by the immortalized HTR-8/Svneo cell line of trophoblasts. These cells interact with EMILIN1 via $\alpha 4\beta 1$ integrin. Purified IgG obtained from the serum of women undergoing recurrent spontaneous abortion interfere with this adhesion. The engagement of this receptor leads to ERK phosphorylation that resulted specific for trophoblast cells and higher than on fibronectin. Using cocultures of EVT and decidual stromal cells we investigated the potential role of MMPs in the haptotactic process and the results obtained by zymographic and western blotting analyses showed an increase of the active form of MMP-2. In conclusion, this study opens additional avenues in the biology of trophoblast and invasion process.

06-05

Whole peripheral blood NK cell cytotoxic activity in immune monitoring of spontaneous abortion in women after IVF/ET

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Introduction: Threatened spontaneous abortion (SA) in women undergoing IVF/ET procedure is today a problem. During pregnancy activation of peripheral blood NK cells is a result of release of Th1 cytokines and mostly represents an echo of local immune disorders and immune reactions in reproductive tissues. Peculiarities of NK cell cytotoxic activity (CA) as an index of SA among other indices was a goal of the study.

Patients and methods: Sensitive method of NK cell cytotoxic activity of whole peripheral blood (WPB) for immune monitoring of SA in women undergoing IVF/ET (n=50) was used. 17 women had threatened SA. NK cell cytotoxic activity of WPB was detected using Eu-labeled K562 target cells. Total NK cell CA and single NK cell CA were determined. Intracellular IFN- γ /IL-4 in CD4⁺, production of IFN- γ , IL-4, IL-10, IL-6, TNF after PHA stimulation of T-lymphocytes in cell culture and cytokine levels in serum, anti-phospholipid (APA) and anti-nuclear antibodies (abs), activating molecules (CD69, HLA DR) on CD8 T-lymphocytes, CXCR3 and CCR4 on CD4⁺ T-lymphocytes, KIR receptor (CD158a) and HLA DR on CD3⁻CD56⁺ cells and CD19⁺CD5⁺ in women after IVF/ET were also calculated.

Results: CA of single NK cell in IVF/ET women with threatened SA was much higher than in other IVF/ET pregnant women with positive gestation. Total CA was elevated too but it was less indicative. Other indices of SA: high level of CD19⁺CD5⁺, high expression of CD69 and HLA DR on CD8⁺CD3⁺, low expression of CD158a on CD3⁻CD56⁺, high ratio of intracellular IFN- γ /IL-4, high production of IFN- γ and TNF in lymphocyte response to PHA and high level of TNF in serum, APA and anti-nuclear abs.

Conclusions: NK cells play important role in mechanisms of SA. CA of single NK cell in WPB may be an index for monitoring of SA in IVF/ET women

because it is indicative and could be used amidst other immune indices.

06-06

Monoclonal antibodies against human trophoblast in female infertility

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During embryo implantation, cell-to-cell interaction of maternal tissues and blastocyst is critical, as well as participation of various adhesive molecules on the cell surface. Monoclonal antibody against trophoblastic cells (TRO-C11) was prepared and used for immunocytochemical detection of antigens on JAR cells and also on normal and abnormal placenta. Expression of the C11 antigen was typical for abnormal placenta and it seems to be connected with differentiation of the cytotrophoblast. Therefore, TRO-C11 monoclonal antibody could be used for detection of placental abnormalities. It is known that the presence of anti-trophoblast antibodies in sera of females can inhibit cytotrophoblast differentiation in the early stage of pregnancy and that way lead to infertility. Further studies can make for the use of TRO-C11 antibody as a positive standard for analysis of antibodies against the trophoblast in the sera of infertile women. Further experimental studies are necessary to show the relationship between the expression of the detected antigen and its role in female infertility.

06-07

Switch on T-cell populations after dendritic cell therapy in the CBA/J \times DBA/2J abortion-prone mouse model

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Introduction: Therapy with syngeneic dendritic cell (DC) diminished the resorption rate (%R) in the

CBA/J × DBA/2J crossbreeding. The underlying mechanisms remain unknown. The presence of TCR $\gamma\delta$, CD8 α , PIBF and PIBF-Receptor (REC)⁺ cells after such therapy is now investigated.

Materials and methods: CBA/J females were inoculated with syngeneic bone-marrow derived DC twice before mating. The studied groups were: 1- no treatment control, 2- mice injected with DC culture medium (DCCM), 3- immunized with DC alone and 4- immunized with paternal DBA/2J antigens pulsed DC, n = 5. The presence of TCR $\gamma\delta$, CD8 α , PIBF and PIBF-REC⁺ cell in placental tissue was analyzed by immunohistochemistry. Depending on the number of positive cells, the data were scored to allow statistical analysis.

Results: DC therapy induced an increase in the number of CD8 α and TCR $\gamma\delta$ positive cells, especially with the therapy employing DC alone. Non-treated mice showed no significant differences in the expression of both CD8 α and TCR $\gamma\delta$ with respect to the animals inoculated with DCCM, while PIBF expression was increased only in the syngeneic DC alone treatment group. DC therapy did not appear to influence the expression of PIBF-REC.

Conclusions: We suggest that DC therapy up-regulates a protective population of TCR $\gamma\delta$ ⁺, CD8 α ⁺, PIBF⁺ cells at the maternal-fetal interface which would be responsible for the corrected %R obtained.

06-08

Which are the risks of intradermal lymphocyte immunotherapy?

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Introduction: Lymphocyte immunotherapy (LIT) has been introduced 20 years ago for women with recurrent pregnancy loss. Moreover, application is discussed for prevention of Rhesus haemolytic disease and for reducing the risk of HIV-1 infection in persons at risk. Serious concerns have been raised regarding LIT safety which are depicted in the light of data from single centre experience using partners lymphocytes intradermally.

Patients and methods: 1) Prospective four week follow-up of 3246 consecutive treatments between 2000 and 2003, 2) Retrospective 2–3 year follow-up

of 3041 IVF couples treated between 1996 and 2002 including 1119 children born, 3) Immunhematologic testing in appropriate cases.

Results: Acute side effects are equivalent to id vaccination. Local blisters are a characteristic feature (6–14%) which is independent of the HLA class I mismatch rate of the couple. They do not resemble graft versus host reaction. There are possible risks for anaphylaxis, posttransfusion purpura, and severe haemolytic disease/ alloimmune thrombocytopenia/ alloimmune neutropenia of the fetus and newborn which could not be verified. The incidence of autoimmune disease (0.15%) and malignancy (0.2%) resembled natural occurrence. The formation of lymphocytotoxic HLA-antibodies is significant (30% after the first LIT, 65% after the second LIT) and might restrict the selection of transplants later in life. On the other hand, this is an effect of pregnancy as well, and antipaternal HLA antibodies might mitigate alloimmune fetal illness.

Conclusion: Risks are low with id LIT and can be minimized by appropriate diagnostic measures and patient selection according to the transfusion guidelines.

06-09

HLA class II and recurrent miscarriage: new aspects

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Previous studies of HLA class II in recurrent miscarriage (RM) have provided contradictory results. HLA-DR3 and to a lesser extent HLA-DR1 have been reported to be susceptibility alleles for RM. We studied linkage disequilibrium between HLA-DRB1 and HLA-G alleles in couples with RM (103 individuals) and control couples (92 individuals). Furthermore, in 85 women with secondary RM we studied the prognostic impact of three class II alleles (HLA-DRB1*15, HLA-DQB1*05 and DRB3*03) that have previously been reported to restrict CD4 mediated immunological reactions against male-specific minor histocompatibility (HY) antigens. Significant linkage disequilibrium was found between both HLA-DR3 and HLA-DR1 and HLA-G*010102 in patients (p < 0.00001 and p < 0.05, respectively). The HLA-G*010102 allele

includes a 14bp sequence insertion in the 3'untranslated region which is associated with low levels of soluble HLA-G and RM. In the prospective study, 22 (44.9%) of the 49 women with secondary RM after a male firstborn positive for HLA-DRB1*15, HLA-DQB1*05 or DRB3*03 delivered a child after the series of miscarriages compared with 29 (80.6%) of the 36 women positive for the same class II alleles who had a female firstborn ($p < 0.001$). Patients negative for the three alleles had similar chances to have a child after RM irrespective of having a male or female firstborn. The previously reported association between HLA-DR3 and -DR1 and RM can partly be explained by linkage disequilibrium with HLA-G alleles associated with RM. Furthermore, the poorer pregnancy prognosis of patients with secondary RM with a male firstborn compared with those with a female firstborn may be explained by a HLA class II restricted response against HY antigens which may initiate a more widespread immune reaction against the fetus.

Poster presentations

06-10

Antiphospholipid antibodies and reproduction: Expression of IgG anticardiolipin antibodies during pregnancy

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Anticardiolipin antibodies (ACLA) are acquired autoantibodies against a phospholipid (cardiolipin) that has been associated with slow progressive thrombosis and infarction in the placenta. Pregnancy loss is one of the most important clinical manifestations associated with the antiphospholipid antibodies. To investigate the significance of ACLA in reproduction, we have determined ACLA IgG plasma levels in the first, second and third trimester of pregnancy. We have examined 73 pregnant women referred to the OB Clinic of the Shiraz University of Medical Sciences. Maternal serum levels of ACLA were measured in primary, secondary and the third trimester of gestation. Concentration of ACLA was measured in all samples (219 samples) by an indirect enzyme linked immunosorbent assay. Only three pregnant women

had moderate positive titers of autoantibodies against cardiolipin (16–24 GPL) (4%). Fourteen pregnant women had low positive titres (11–15 GPL) of ACLA IgG tending to normal levels (<10 GPL) in the first and the third trimester respectively. All other pregnant women had normal titers of ACLA in these periods. These results demonstrate that maternal serum ACLA levels and/or differential expression of these autoantibodies over 96% of Iranian pregnant women were in the normal range during this period.

06-11

Antibodies to cardiolipin in preeclampsia

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Preeclampsia is the most common hypertensive disorder during pregnancy and represents life threatening situations for the mother and fetus. It remains a leading cause of maternal and fetal morbidity and mortality. Although new mechanisms and insights are being found continually, the etiology of preeclampsia remain unknown. Preeclampsia is diagnosed clinically by increased blood pressure accompanied by proteinuria and is associated with impaired trophoblast invasion. Antiphospholipid antibodies are widely a cause of obstetrical complications such as preeclampsia. The objective of this study was to test the anticardiolipin (ACLA) in preeclamptic women compared with normal pregnancy. This study comprised 121 pregnant women in the third trimester (32–40 weeks gestation) who attended the OB/GYN Clinic of Shiraz University of Medical Sciences. They were divided into two groups: 60 pregnant women and 61 preeclamptic patients. Serum concentrations of anticardiolipin antibodies were measured in these two groups and in 40 non-pregnant control samples. Concentration of IgG ACLA were measured by an indirect ELISA immunoassay. Serum concentration of IgG ACLA was significantly higher in women with preeclampsia compared with non-pregnant and normal pregnant controls. None of the patients had high positive titers of ACLA (>40 GPL). Moderate positive titer of antibodies (15–20 GPL) were found in 11.4% of preeclamptic patients. According to these results, there is evidence of increased production of serum antibodies against

cardiolipin in Iranian women with preeclampsia, and it may be associated with this obstetrical complication.

06-12

Increased NK cell cytotoxicity and IL-2 production in recurrent miscarriage

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It has been postulated that a proportion of recurrent miscarriage (RM) might be due to immune causes. The aim of this study was to determine the NK cytotoxicity and cytokine production in peripheral blood of patients with a history of RM and any correlation between cytokine production and NK cytotoxicity. This study was carried out on three different groups, (group I, n = 21) patients with RM at the time of 3rd or more abortion, (group 2, n = 21) patients with a history of RM (with at least 3 pregnancy losses), 3 months after their last abortion, (group 3, n = 21) normal pregnant women. The NK cytotoxicity in peripheral blood using flow cytometry and cytokine production by stimulated peripheral blood mononuclear cells (PBMC) was determined. Group I and Group II showed significantly higher NK cytotoxicity than Group III ($P \leq 0.045$ and $P \leq 0.002$ respectively). The correlation between production of IL-2 and NK cytotoxicity was significant ($R \leq 0.350$, $P \geq 0.001$). Group I and Group II had significantly higher levels of IL-2 than Group III ($P = 0.002$ and $P = 0.001$ respectively). In Group III the production of IL-10 by PBMC was higher than both Group I and II ($P = 0.002$ and $P = 0.001$ respectively). Increased NK cell cytotoxicity and high level of IL-2 may be considered as risk factors of RM.

06-13

Molecular genetic analysis of HLA-A, B and DR alleles gene detection in a couple with recurrent spontaneous abortion (RSA)

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Recurrent spontaneous abortion (RSA) is defined as three or more miscarriage in first trimester of pregnancy and postulated to be due to several factors including immunogenetic mechanisms. One half of the fetus human leukocyte antigen (HLA) type is contributed by mother and the other half by father. When a woman becomes pregnant, her bodies immune system usually recognizes the fathers HLA as different from her own, and the white blood cells in her uterus produce protective blocking antibodies. These antibodies coat the babies' cells and protect the fetus from mother's killer cells. We obtained peripheral blood samples from couples with three or more abortions and multiparous. DNA was extracted from peripheral blood cells by standard phenol-chloroform procedures. Molecular HLA typing for more than 160 alleles of HLA-A, B and DR was carried out by using polymerase chain reaction (PCR-SSP) with appropriate primers. PCR products were analyzed on an agarose gel and visualized by UV following ethidium bromide staining. The results show that although some HLA alleles are more frequent in some groups, generally, there was no significant difference between HLA sharing. HLA-B7 was present in all multiparous couples while it was absent in most aborters. These results suggest that HLA sharing isn't a reliable factor for predicting pregnancy outcome and it is better to focus on special alleles such as HLA-B7. High frequency of HLA-A1/36,24(9) in male and HLA-A2,24(9) in female, HLA-Bw6, 51(5) in male and HLA-Bw6, 51(5) in male and HLABw6, 72(70) in female, HLA-DR52, 13.4 in male and HLA-DR52,13.3 in female. In conclusion, these results suggest that if father's HLA is too similar to mother her cells may not recognize differences that are vital to the production of blocking antibodies.

06-14

Recurrent pregnancy loss as a sole manifestation of systemic lupus erythematosus (SLE)

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There is general agreement that Lupus Anti-Coagulants or Anti-Cardiolipin in SLE women are linked with recurrent pregnancy loss. In a review of the literature it was found that the incidence of preg-

nancy loss was 60% and 59% for SLE patients with Lupus Anti-Coagulants or Anti-Cardiolipin respectively, and 13% and 5% for SLE patients without Lupus Anti-Coagulants or Anti-Cardiolipin respectively. Pregnancy loss can occur in the first, second or third trimester of pregnancy. Even in the case of a successful pregnancy, Phospholipid antibody have been associated with other unfavorable outcomes such as preeclampsia, HELLP syndrome, chorea gravidarum, intrauterine growth retardation, stillbirth, preterm births, and thrombosis. In spite of considerable interest in these autoantibodies, the mechanism(s) implicated in the association between Phospholipid antibody and fetal wastage remains unclear. There are still doubts whether Phospholipid antibody has a pathogenic potential on their own, or whether they are epiphenomena. The cause of fetal wastage is thought to be thrombosis (or eventually vasculopathy) in placental and decidual vessels. A large number of potential pathological mechanisms inducing thrombosis have been proposed for Phospholipid antibody such as increased tissue factor generation, inhibition of the thrombomodulin-protein C pathway (the placenta is rich in thrombomodulin), interference with the prostacyclin-thromboxane balance, inhibition of the fibrinolytic system and so on, as well as combinations of them. Based on the clinical and laboratory heterogeneity of Phospholipid antibody, it is logical to assume a similar diversity for the pathophysiology of pregnancy loss. In this article a 31 year old woman will be presented without any clinical criteria of SLE. She experienced five consecutive fetal losses since 22-years of age. Laboratory evaluation revealed high titer ANA and Anti ds-DNA, associated with low complement levels. There was a history of ITP in her past history when she was eighteen. Neither dermatologic nor renal and CNS manifestations of SLE was detected.

06-15

Lack of association between the TGF- β 1 gene polymorphisms and recurrent spontaneous abortion

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Transforming growth factor- β 1 (TGF- β 1) is produced by T regulatory lymphocytes (Treg), which play an important role in the physiology of pregnancy. Several polymorphisms of the TGF- β 1 gene (*TGFBI*) have been reported, some with an important correlation with TGF- β 1 production and disease severity. We performed an association study between *TGFBI* polymorphisms and recurrent spontaneous abortion (RSA). We first used a PCR-RFLP method to detect three known *TGFBI* cSNPs (coding single nucleotide polymorphisms) among 111 RSA and 110 normal control women from southern Iran, such as 29 T \rightarrow C (Leu 10 Pro), 74G \rightarrow C (Arg 25 Pro) and 788C \rightarrow T (Thr 263Ile), and compared their frequencies between the two groups of subjects. To confirm results of the RFLP study and to identify new SNPs in the RSA women, we then sequenced their DNA samples for seven exons and adjacent intronic regions of *TGFBI*. Consequently, ten SNPs were detected, one (-14G \rightarrow A) was located in the upstream region of exon 1, three in exons (two in exon 1 and one in exon 5) and six in intronic regions. Two (IVS 5+18G \rightarrow C and IVS 6+910G \rightarrow A) of the ten SNPs were novel. Statistical analysis on the frequency of six most frequent SNPs including the three cSNPs, as well as on the frequencies of genotypes and 13 haplotypes regarding the 6 SNPs, revealed no significant difference between RSA and control women. Therefore, it is less likely that exonic and adjacent intronic polymorphisms of *TGFBI* are associated with RSA.

SPECIAL INVITED LECTURE

07-01

Developments in vaccine design

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Passive immunization: Preformed antibodies are appropriate for several circumstances and increasingly provide a therapeutic option in cancer and cytokine-mediated disorders such as rheumatoid arthritis and Crohn's disease. They can be derived from many sources: recombinant IgG, Fab, scFv, human and llama V_H can be selected from phage libraries, plants can produce monoclonals and defensins in bulk while commensal bacteria such as lactobacilli and *Strep. gordonii* can deliver Fab fragments.

Subunit vaccines: Whole organisms have a multiplicity of antigens, some of which are not protective, may induce hypersensitivity or might even be frankly immunosuppressive. It makes sense in these cases to use purified components. There is greatly increased use of recombinant DNA technology to produce these antigens. Expression in bananas and potatoes provides a very cheap way of achieving oral immunization in the developing world. There is increasing emphasis on immunization with the gene(s) encoding the desired subunit vaccine. These can be introduced through insertion in attenuated living organisms such as vaccinia or BCG. Increasingly the naked DNA alone or coated onto microparticles can be injected directly. The advantages are stability, ease of production and cheapness. Epitope-specific vaccines based on conserved structures have the advantage that they can provide broad protection and may avoid the possible deleterious effects of other epitopes (autoimmunity, T-downregulation, original antigenic sin, escape by mutation of immu-

nodominant epitopes) when certain whole antigens are used for immunization. Epitope-specific vaccines can be based on peptides, internal image anti-idiotypes or epitope-loss mutants. Peptides can mimic linear B and T-cell epitopes; where a T-cell vaccine is needed for rapidly mutating viruses such as HIV, a series of peptide epitopes can be linked together. Progress in producing surrogate discontinuous peptide epitopes will be described. Carriers such as tetanus toxoid or mycobacterial heat shock proteins are usually essential to evoke an antibody response to the peptide (or to a carbohydrate). Epitope-loss mutants have undesirable epitopes replaced but still fold correctly to produce the wanted discontinuous B-cell epitope(s).

Adjuvants: Adjuvants work by producing depots of antigen, and by activating antigen-presenting cells usually through their Toll-like receptors.

Delivery: New methods of delivery include linking the antigen to small lipid membrane vesicles (liposomes) or a special glycoside matrix (Iscom). These delivery particles can be furnished with many factors which improve their immunogenicity and flexibility. Theoretically one could build in several antigens into the same particle, adjuvants such as MDP and MLA, cytokines to influence lymphocyte subset responses and molecules such as cholera toxin B to target particular sites in the body. Intranasal and transdermal routes of immunization are finding increasing favour. Antigens built into biodegradable polymers of varying half-life can provide single-shot vaccines which mimic a conventional course of immunization requiring several injections.

Cancer vaccines: The development of cancer vaccines based on reproductive and other hormones and a surface carbohydrate antigen for example, is being actively pursued.

Additional abstracts

The control of embryo implantation. Studies of gene expression, protein profiles/function at the uterus-embryonic level: cellular and molecular developmental events at the fetomaternal interface

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Embryo Implantation Control (EMBIC) is a European Network of Excellence partially financed by the European Commission through the FP6 program. Today, this Virtual European Laboratory is composed of 170 researchers from Austria, Belgium, Croatia, France, Germany, Hungary, Italy, Spain and the United Kingdom.

The **EMBIC** scientists aim to understand the factors involved in embryo implantation. A multidisciplinary approach has been implemented to:

- a) Identify and characterise the factors released by the pre-implantation embryo and their role in implantation success
- b) Identify the uterine modifications and molecular and cellular signals that contribute to the development of a "receptive" uterus for the implanting embryo
- c) Understand the biological mechanisms that permit the embryo and mother to successfully coexist throughout pregnancy

EMBIC will also strengthen the European Research Area by:

- a) facilitating mobility, communication and training among the EMBIC members.
- b) attracting young researchers and physicians to the fields related to **EMBIC's** scientific goals. We propose to hold each year a leading Summer School on Embryo Implantation and provide exciting training opportunities.

Our bench-to-bed strategy will allow us to translate our results in the near future to areas such as improvements in embryo culture media and new diagnostic tools and, later, innovative treatments to increase the embryo implantation rate.

EMBIC is open to new opportunities that will help us to improve our research and clinical capacities.

The complement regulatory protein CD55 is expressed on the inner acrosomal membrane of human spermatozoa

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CD55, a glycosylphosphatidylinositol (GPI)-anchored inhibitor of complement has previously been shown to be surface expressed on human spermatozoa, whereas another complement inhibitor CD46 is only expressed on the inner acrosomal membrane (IAM) which becomes surfaced exposed after the acrosome reaction (AR) when sperm assume fertilisation-competence. Dual labelling immunofluorescence & confocal microscopy using fresh AR-spermatozoa has shown that CD55 is in fact primarily expressed on the IAM, more than on the plasma membrane, although in a patchier pattern than for CD46. Western blotting of spermatozoal CD55 revealed a 50–55 kDa protein, some 15–20 kDa lower than that expressed by other cell types and removal of N- and O-linked carbohydrates had no effect on the apparent size of spermatozoal CD55. Membrane rafting & release of CD55 could have influenced previous results. CD55 may play a role in the signalling machinery for the AR; moreover, significant co-expression of CD55 with CD46 on the IAM suggests some functional cooperation at this site.

Immunocontraception – Is it feasible in humans?

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Approximately 50% of human pregnancies are unintended, and unfortunately often unwanted. Clearly, increased use of contraception is desirable. There is

also a need for a wider choice of family planning options. One possibility is the development of contraceptive vaccines. Both gametic and hormonal antigens have been proposed as vaccine candidates. Zona pellucida antigens from the oocyte, and a number of sperm-associated antigens, can elicit antibodies capable of blocking fertilization. Hormones that can be targeted include human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH) and gonadotropin-releasing hormone (GnRH). The vaccines that have progressed furthest are those based on hCG, a hormone required for the maintenance of pregnancy. hCG has also been used in anti-cancer vaccines because this 'pregnancy-specific' hormone is also aberrantly expressed by a number of tumors. Whether used in a contraceptive or a cancer setting, these vaccines have demonstrated minimal side effects and therefore, in the limited trials to date, appear to be safe. Furthermore, phase II clinical trials of an hCG-based contraceptive vaccine produced by Talwar and colleagues have established the efficacy of this approach in preventing pregnancy. Efficacy is dependent on the vaccine eliciting a sufficient level of antibody to neutralize the biological activity of the hCG. The contraceptive effect is reversible, as normal pregnancy can proceed in the absence of booster immunizations once the neutralizing antibodies are naturally catabolized. If long-term safety can be established in larger cohorts of patients, then immunocontraception would be an extremely attractive additional family planning option.

domain composed of eleven zinc fingers (ZF). CTCF has features of a tumour suppressor and is involved in regulation of multiple cellular functions. They include transcription activation and repression; regulation of cell proliferation, differentiation and apoptosis; control of insulator elements and imprinting. A paralogue of CTCF, designated BORIS for Brother of the Regulator of Imprinted Sites, has been recently identified. BORIS contains the DNA-binding domain identical to CTCF, but different N- and C-terminal domains. BORIS is expressed only in the testis and thus can be considered as a new member of the cancer-testis antigen family (Loukinov et al, Proc. Natl. Acad. Sci. USA, 2002, 99: 6806–6811; Klenova et al, Seminars in Cancer Biology, 2002, 449:1–16). Our previous results demonstrated that during male germ-line development CTCF and BORIS are expressed in a mutually exclusive manner; this may be important for epigenetic reprogramming occurring in these cells during development. The aim of this study was to investigate in more detail the expression patterns of CTCF and BORIS during mouse spermatogenesis. Western analysis of purified stage-specific spermatogenic cells (stages I–XIV) dissected from mouse testes has revealed low CTCF levels in cells at all stages. BORIS protein has also been detected at all stages, however significant elevation of BORIS expression was noted in pachytene spermatocyte (stages VII–VIII). Possible implications of these findings will be discussed in relation to the existing hypothesis of the functional role of mutually exclusive expression of CTCF and BORIS.

Expression of a transcription factor, CTCF, and its paralogue, BORIS, during mouse spermatogenesis

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CTCF is an evolutionarily conserved and ubiquitous transcription factor containing a DNA-binding

Reduced semen quality in chronic prostatitis patients that have cellular autoimmune response to prostate antigens

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Introduction: The relationship between chronic prostatitis (CP) and fertility has been controversial for decades. We have previously shown the presence of an autoimmune response against prostate antigens (PA) in chronic prostatitis patients. Our main goal

was to investigate whether CP could have deleterious effects on semen quality.

Patients and methods: We analyzed the presence of autoimmune responses against PA either in patients with chronic infectious prostatitis (CIP) or chronic non-infectious prostatitis (CNIP) and analyzed their semen quality and levels of seminal inflammatory cytokines.

Results: We demonstrated the presence of lymphocytes able to proliferate in response to human PA (PSA and PAP) only in a group of patients with CNIP. Elevated levels of $\text{INF}\gamma$ were detected only in supernatants samples from patients with positive proliferative response to PA. Interestingly, these patients showed striking alterations on their semen quality and increased levels of inflammatory cytokines (IL-1 and $\text{TNF-}\alpha$) in their seminal plasma.

Conclusions: We found that $\text{INF}\gamma$ -secreting lymphocytes specific to PA are detected in 34% of the patients with CNIP and an association with the marked alterations observed in semen. These results argue in favor of the importance of prostate functionality for the quality of the semen.

Pathogenic consequences on semen quality of an autoimmune response against prostate antigens

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Introduction: Previously, we have found alterations in the semen quality of patients bearing chronic prostatitis with an autoimmune response against prostate antigens. In this study we analyzed the presence of sperm alterations and the involved mediators in an experimental model of autoimmune prostatitis.

Materials and methods: Autoimmune prostatitis was induced in 2 groups of Wistar male rats, by the injection either of a prostatic extract of proteins (EP) or prostatein (PSBP). A control group was included (C). The autoimmune response and several sperm parameters, in semen and epididymal sperm, were studied at different time-points.

Results: Animals from groups EP and PSBP developed a positive cellular and humoral autoimmune

response against prostate antigens. They also showed striking alterations in semen (reduced sperm density, motility, viability) when compared to group C. No alterations in epididymal sperm were observed. We also found significantly increased levels of apoptosis in seminal sperm from both groups of animals (EP and PSBP) but not in epididymal sperm. Citric acid, the prostate biomarker, was found diminished in these animals. Besides, we detected increased levels of nitric oxide and $\text{TNF-}\alpha$ in seminal plasma from these autoimmune animals, evidencing local inflammation.

Conclusions: These results support the hypothesis that an autoimmune response against prostate can significantly alter the sperm quality on an individual, thus compromising fertility.

HLA-G1 protects Jeg-3 choriocarcinoma cells against NK cells

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Problem: Trophoblast cells and choriocarcinoma cells escape from NK cell killing by interaction of HLA-G with killing inhibitory receptors. The role of HLA-G isoforms is not yet exactly revealed.

Methods of study: RNAinterference (RNAi) was applied to Jeg-3 cells to knock down total HLA-G and HLA-G1 and soluble HLA-G1 (HLA-G5). Small interfering RNA (siRNA) and scrambled oligonucleotide controls were self-designed. Due to homologies in the HLA-G gene structure, siRNA to knock down further single isoforms seem to be not realizable. HLA-G knock down was analyzed by Western blot and flow cytometry. NK cells were isolated from peripheral blood of healthy donors. For cytotoxicity assays, Jeg-3 cells were stained with CFSE and cocultured with NK cells. Death rate was measured by flow cytometry.

Results: HLA-G1 knock down was successfully performed. Increase of cytotoxicity after HLA-G1 knock down was similar to that after total HLA-G knock down.

Conclusion: Among the different HLA-G isoforms, HLA-G1 plays a key role for protection of trophoblastic cells from NK cell cytotoxicity.

Leukemia Inhibitory Factor (LIF) and Interleukin-6 Induced Intracellular Signalling in Jeg-3 choriocarcinoma cells

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Problem: Leukemia Inhibitory Factor (LIF) is major regulator of the natural course of pregnancy. Invasion of trophoblast cells requires a fine tuning, which is fundamental for correct placentation. This tuning is regulated on intracellular level and influenced by LIF. Several signal transducers and their suppressors, known from former studies and tumor invasion, are expected to be involved.

Methods of study: RNAinterference (RNAi) was applied to Jeg-3 cells to knock down STAT3, SOCS3

and STAT6. Small interfering RNA (siRNA) and scrambled oligonucleotide controls were self-designed. Jeg-3 cells were stimulated with LIF, IL-4 and IL-6. Expression and phosphorylation of factors were analyzed by Western blots. Proliferation was measured by using a colorimetric assay, invasion by matrigel assays.

Results: Knock down of STAT3 reduced LIF induced proliferation and invasion, whereas SOCS3 knock down increased IL-6 induced Tyr705 phosphorylation of STAT3 simultaneously with proliferation. STAT6 RNAi had no influence on invasion, but IL-4 induced proliferation was inhibited.

Conclusion: LIF and IL-6 use STAT3, SOCS3 and STAT6 to regulate proliferative and invasive capacities of Jeg-3 choriocarcinoma cells, which may be regarded as a model for trophoblast cells.