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Cytokine expression at interface: a longitudinal micro array

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► **To cite this version:**

G rard Chaouat, Nathalie Coqu , B. Hennuy, S. Dubanchet, I. Bataillon, et al.. Cytokine expression at interface: a longitudinal micro array. 4. EMBIC Summer School entitled "Advances in embryo Implantation and pregnancy", Jun 2008, Barcelone, Spain. 10.1111/j.1600-0897.2007.00495.x . hal-01197579

HAL Id: hal-01197579

<https://hal.science/hal-01197579v1>

Submitted on 3 Jun 2020

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MAIN SESSION 1: GAMETE IMMUNOLOGY

MS1-1

Biological and immunological significance of CD52 in Reproduction

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Human CD52 is a highly glycosylated molecule with a small core peptide exclusively expressed on lymphocytes and mature sperm via the GPI anchor. In male reproductive tracts (mrt), it is secreted mainly from the epididymis and inserted into the sperm membranes via the GPI anchor during their passage through the epididymis. It was found that the male mrt-CD52 was a target antigen of a human monoclonal antibody (Mab H6-3C4) generated from an infertile woman. The Mab H6-3C4 showed a strong sperm-immobilizing activity with complement (C') and specifically recognized the N-linked carbohydrate epitope of sperm CD52 but not lymphocyte CD52. mrt-CD52 purified from human seminal plasma showed C' inhibitory effect on hemolytic assay and C'-dependent sperm immobilization test in the same way as GPI anchored C' regulatory molecules of CD59 on sperm. Mouse Mab 1G12 reacting to human mrt-CD52 strongly inhibited fertilization by human sperm before and after capacitation and acrosome reaction in a zona-free hamster egg penetration test. Mouse CD52 was similar to human CD52 in its biological and immunological characteristics. The molecule was exclusively expressed on mature sperm and lymphocytes via the GPI anchor and highly-glycosylated with the N-linked and O-linked carbohydrates. Male and female mice immunized with mouse mrt-CD52 molecules produced auto- and iso-antibodies against the cognate antigen yielding antisera with C'-dependent mouse sperm immobilizing activities. Furthermore, surprising our recent results indicated that CD52 molecules were found in cumulus-oocyte complexes in mice and humans, suggesting some roles of CD52 in the final maturation of male and female gametes.

MS1-2

Pre-patterning of the mammalian oocyte revealed by KH domain RNA binding proteins MOEP19 and Ecat-1

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Problem: Despite evidence that large oocytes from species such as frogs and molluscs contain regionally distributed RNAs and RNA binding proteins that show asymmetries and play a role in determining cell fates there is little evidence in mammalian oocytes for an informative molecular mosaicism in the ooplasm that might predict later developmental events.

Methods of Study: Proteomics was employed to identify novel KH domain containing RNA binding proteins whose localization was examined in oocytes and early embryos.

Results: MOEP19 and Ecat-1 proteins were first detected in primary follicles and accumulated during oogenesis as oocytes expanded but showed no evidence of being translated in ovulated eggs. These proteins localized in the ovulated egg and early zygote as a symmetrical spherical cortical domain underlying the oolemma, deep to the zone of cortical granules. These proteins remained restricted to a cortical cytoplasmic crescent in blastomeres of 2, 4 and 8 cell embryos, and did not distribute into regions underlying cell contacts. In morulae, MOEP19 and Ecat-1 remained at the apex of outer, polarized blastomeres and were undetectable in blastomeres of the inner cell mass. In early blastocysts, MOEP19 and Ecat-1 localized diffusely throughout the cytoplasm of both mural and polar trophoctoderm and the inner cell mass, and their concentrations declined until disappearing from all but a single cell in expanded late blastocysts. When blastomeres of 4-8 cell stages were dissociated, the polarized MOEP19/Ecat-1 domain assumed a symmetrically spherical localization, while overnight

culture of dissociated blastomeres resulted in formation of embryoid bodies in which polarity of the MOEP19/Ecat-1 domain was re-established at the blastomere apices.

Conclusion: The persistence during early development of the MOEP 19/Ecat-1 cortical oocyte domain as a cortical crescent in blastomeres suggests an intrinsic pre-patterning of the oocyte that is related to the apical-basolateral polarity of the embryo.

MS1-3

The function of CD46 on sperm: man or mouse?

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Problem: CD46 is a cell surface complement regulatory (CReg) glycoprotein. However, on sperm, it is expressed on the acrosomal membrane (AM) and becomes surface exposed only after the acrosome reaction (AR) when sperm become fertilization-competent. The function(s) of spermatozoal CD46 are uncertain.

Method of study: PCR, RT-PCR, 5'- and 3'-RACE, immunocyto/histochemistry, AR rate measurement using PNA lectin.

Results: Human CD46 is broadly expressed. However, on sperm AM, it is expressed in a lower Mr isoform. Antibodies to the first short consensus repeat (SCR1) domain inhibit fertilization by human sperm. In contrast, CD46 is not a systemic CReg protein in laboratory rodents but instead is solely expressed by sperm. CD46 knockout mice are fertile and have an accelerated AR rate. Wood mice (*A. sylvaticus*) show promiscuous behaviour, with pronounced competition for individual mating success. We have demonstrated that wild-caught *A. sylvaticus* produce unique testicular CD46 transcripts lacking exons 5-7 or 6-7, together with an extended AU-rich 3'- and often truncated 5'-untranslated region. Monoclonal and polyclonal anti-rodent CD46 antibodies (including rabbit antibody to a 14-mer peptide predicted from the translated *A. sylvaticus* CD46 SCR1 mRNA sequence) were studied on epididymal

sperm and testis sections. *A. sylvaticus* sperm fail to express any CD46 protein, unlike balb/c controls. These splicing events and absence of CD46 protein expression occur in other wild-caught *Apodemus* (*A. microps* and *A. flavicollis*) but not *Mus* (*M. musculus* and *M. domesticus*). Failure to express CD46 protein in *Apodemus* correlated with a consequent accelerated AR rate compared to *Mus*.

Conclusions: Human sperm CD46 may function primarily at the level of egg interaction. In rodents, expression has been lost from cells other than sperm and, in *Mus*, CD46 may instead function primarily in providing acrosomal stability. The CD46 gene is redundant in *Apodemus*, trading this CReg protein expression for an accelerated AR.

MS1-4

Fertilization in humans: Dissecting the complexity of zona pellucida matrix and functional relevance of its constituents

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Problem: Human zona pellucida (ZP) matrix is composed of 4 glycoproteins designated as ZP1 (638 aa), ZP2 (745 aa), ZP3 (424 aa) and ZP4 (540 aa). Non-availability of purified human zona proteins from the native source has hampered critical appraisal of their role during fertilization.

Method of Study: To delineate the functional relevance of the human zona proteins, our group has expressed these both in prokaryotic and eukaryotic expression systems. Purified fluorescence-tagged recombinant proteins have been employed to investigate their binding characteristics to the human spermatozoa. In addition, their ability to induce acrosomal exocytosis has also been investigated.

Results: Both *E. coli*- as well as baculovirus-expressed recombinant ZP3 and ZP4 binds to the anterior head of the capacitated human spermatozoa. Recombinant ZP2 failed to bind to the capacitated spermatozoa and showed binding to the equatorial region of the acrosome-reacted spermatozoa. Double labeling experiments revealed simultaneous binding of both ZP3 and ZP4 to the capacitated spermatozoa. Similar binding

profiles were obtained with native human zona proteins in an indirect immunofluorescence assay using rabbit polyclonal antibodies. Baculovirus-expressed recombinant ZP3 and ZP4 induce acrosomal exocytosis in the capacitated spermatozoa where as both *E. coli*-expressed proteins failed to do so. Recombinant proteins deficient in N-linked glycosylation showed marked reduction in their ability to induce acrosomal exocytosis.

Conclusions: Thus, glycosylation of the human zona proteins is not essential for their binding to the spermatozoa but is critical for inducing acrosomal exocytosis. In humans, more than one zona protein may participate in inducing acrosomal exocytosis.

MS1-5

Possible role of zona pellucida proteins in ovarian follicle development

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Problem: The zona pellucida (ZP) is an extracellular matrix surrounding growing oocytes, ovulated oocytes and preimplantation embryos. In mice, the ZP is composed of three glycoproteins called ZP1, ZP2 and ZP3. ZP2 and ZP3 genetically-knockout mice are infertile due to defective folliculogenesis. The ZP proteins are therefore thought to be important factors for folliculogenesis. This study examines the possible roles of the ZP in ovarian function using short interfering (si) RNAs and anti-ZP antibodies in animal experiments.

Method of study: Oocyte-granulosa cell complexes (OGCs) were collected by mechanical dissection and collagenase treatments from juvenile mice. Two siRNAs were injected into the oocyte through the granulosa cell layers and cultured for 5 days. The survival rates and diameters of the oocytes were estimated. When examining the effects of antibody, anti-ZP2 or anti-ZP3 antisera were added to the medium during 7 day-culture and assessed OGC development, oocyte maturation, fertilization and embryo growth.

Results: Oocytes injected with ZP1-, ZP2-, and ZP3-siRNA showed less survival rates compared to the

control group. In antibody experiment, both of the anti-ZPA and anti-ZPC antibodies did not affect follicular cell growth but impaired oocyte maturation, fertilization and embryo growth. It suggested that the oocytes reduced their quality when they had been exposed to the antibodies in follicle development.

Conclusions: These results indicated that ZP plays an important role in oocyte development. When autoantibody produces and binds to the ZP, it may impair physiological ovarian function. Therefore, anti-ZP antibody is a possible immunological factor to premature ovarian failure.

MS1-6

Molecular analysis of IL-1-IL-18 system in human gonad

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Spermatogenesis is a process which is regulated by endocrine and auto/paracrine factors, Under pathological conditions, such as testicular infection, inflammation or tumor growth, the levels of cytokines in the male gonad could be imbalanced and thus to disrupt the homeostasis.

Method of study: Complex studies using real-time PCR, Northern blotting, computer modeling and other molecular techniques for study of IL-1-IL-18 gene family and its products have been used to investigate the homogenated tissue samples originated from normal testis, spermatogenetic arrests and testicular tumors.

Results: The analysis of mRNA expression in tissue samples from testis with maturation arrest, Sertoli cell only syndrome (SCOS) and obstructive azoospermia showed the inversely related balance between IL-1 α and IL-1RA. The maturation block of spermatogenesis (azoospermia) was connected with almost equal expression of both these genes. When spermatogenesis was close to normal (obstructive azoospermia) the expression pattern resembled the physiological status of testis (prevalence of IL-1RA mRNA over IL-1 α). We created a quotient value ($Q_{IL-1/RA}$) for potential diagnostic tool. The Kruskal-Wallis test showed significant differences between $Q_{IL-1/RA}$ measured in pathological [maturation block

($p < 0.003$) and SCOS ($p < 0.0003$) and physiological condition. The quantitative analysis of mRNA for the range of genes from IL-1-IL-18 family in testicular tumors revealed raised level of anti-inflammatory genes (IL-1RA and IL-1RII) and surprisingly of genes connected to apoptosis, IL-18 and ICE.

Conclusions: We have found that all analyzed genes are expressed both in physiological and pathological male gonad but the profile of transcription is distinct.

MS1-7

Small interference RNA-mediated knockdown of sperm associated antigen 9 involved in sperm egg interaction

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Recently, we reported a novel testis-specific sperm associated antigen 9 (SPAG9) protein, a new member of the JNK-interacting protein family, having a role in MAPK signaling pathway. In the present study, we compared the efficiency of two independent SPAG9 specific small interfering RNA (siRNA) constructs, BS/U6/spag9 and BS/U6/spag9-I, to ablate the SPAG9 expression in mammalian cells. A positive correlation between the ratio of target gene versus siRNA and the suppression of SPAG9 expression was observed. Further, the co-transfection of BS/U6/spag9 with pcDNA-SPAG9 and pFlag-CMV2-JNK-3 resulted in specific suppression of SPAG9 without affecting JNK-3 expression. The present investigation will eventually extend the application of SPAG9 siRNA in *in vivo* targeting experiments that aim to define the SPAG9 functional genomics in reproductive biology.

Acknowledgement: This work was supported by grants from Department of Biotechnology, Government of India and Mellon foundation and CONRAD, USA. Email: anil@nii.res.in

MS1-8

Th1 and Th2 cytokines after in vitro PBMC stimulation with sperm cells/ antigens in infertile women and men with sperm antibodies

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Problem: To compare the clinical occurrence of serum sperm antibodies in infertile women and men, and cellular response as different spectra of cytokines produced after in vitro peripheral blood mononuclear cells (PBMC) stimulation with sperm cells/antigens. Methods of study: PBMC isolated from group of infertile women (19) and men (10) with antisperm antibody, group of fertile healthy women (5), virgo (5) and fertile healthy men (4) were stimulated in vitro for 3 and 5 days with sperm cells of healthy donors. The culture media were subjected to multiple cytokine analysis using RayBio Human Cytokine Antibody Arrays III, enabling to detect 40 cytokines at once. Antisperm antibodies were determined by indirect mixed antiimmunoglobulin reaction test (i-MAR) in IgG (26), and IgM (3).

Results: Individual variability in the presence and concentration of cytokines within PBMC samples was found. In infertile, sperm-antibody positive women, significantly higher levels of some cytokines than in control fertile women were detected e.g. GM-CSF, eotaxin, IL-10, IL-12p40, IL-6, IL-8, MCP2. In infertile men with positive serum sperm antibodies, significantly higher levels of eotaxin 2, IL-6, IL-8, MCP1, MIP1 β and IL-10 in contrast with control group were found.

Conclusions: Our results suggest that activation of the immune system by sperm cells/antigens in infertile patients leading to production of antisperm antibodies as well as to production of various spectra of cytokines could be responsible for impaired fertilization process and/or early embryo development. Acknowledgment to Charles University (grant N. MSM 0021620812).

MAIN SESSION 2: MHC AND REPRODUCTION

MS2-1

Soluble HLA-G and control of angiogenesis

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Problem: During human early pregnancy, endovascular trophoblast partly replaces endothelial cells lining the spiral arteries, leading to vascular uterine remodeling. Knowing that these extravillous trophoblast cells produce soluble HLA-G, we hypothesized that this molecule could play a role in this process. Lack of HLA-G production in preeclampsia, characterized by a defect in uterine vascular remodeling, favoured this hypothesis.

Method f study: We set up a number of *in vitro* and *in vivo* experiments to demonstrate that soluble HLA-G molecules affect endothelial cell activity (Fons *et al.* Blood 2006, 108; 2608–2615).

Results: We found that a recombinant soluble HLA-G1 (G5) inhibited *in vitro* proangiogenic factor-activated endothelial cell proliferation, migration, and tubule-like formation. We demonstrated that these effects occurred through the binding of soluble HLA-G to CD160, a glycosylphosphatidylinositol-anchored receptor, and via a caspase-dependent apoptotic pathway. We also demonstrated that soluble HLA-G1 blocked *in vivo* rabbit corneal neoangiogenesis.

Conclusions: In addition to their immunoregulatory functions, soluble HLA-G molecules also inhibit activated endothelial cell activity. How this influences uterine vascular remodeling *in situ* remains to be demonstrated. This work was partly supported by EMBIC.

MS2-2

Nonclassical HLA class I and the immunology of pregnancyA Ishitani¹, N Sageshima¹, N Lee², K Hatake¹, D Geraghty²

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The nonclassical HLA class I HLA-E, -F, -G are distinguished from classical class I by specific

expression patterns and by low allelic polymorphism. Whereas HLA-E is expressed wherever HLA class I is expressed, and is complexed with nonamer peptide derived from class I protein, HLA-G expression is primarily restricted to placental trophoblasts while HLA-F expression has been detected on B-LCL and some monocyte cell lines. We recently showed HLA-E and HLA-F are also expressed on the surface of extravillous trophoblast, where HLA-E is complexed with a unique HLA-G derived nonamer. Placental trophoblasts that have invaded the maternal decidua are the first cell type to show expression of all three nonclassical class I antigens. Extending this, we examined the timing of expression on trophoblasts, and found HLA-F protein on trophoblasts from mid to term of gestation. Examination of other tissues showed HLA-F was expressed on some tumor cells and on lymphocytes activated *in vitro*. Towards functional analysis, we used HLA-E tetramer complexed with different HLA derived nonamers to obtain evidence that the HLA-E/G nonamer complex may bind a unique receptor and further propose that this binding may lead to the accumulation of NK cells specific to the decidua. Our ongoing studies are framed by the expression of all three proteins on the surface of extravillous trophoblasts suggesting that they contribute collectively to enable the growth of the developing child.

MS2-3

The role of MHC class I in establishment and maintenance of pregnancy in cattleS Ellis, I Sargent², T Fair³

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Major histocompatibility complex (MHC) class I genes are involved in the establishment and maintenance of pregnancy. Expression of non-classical MHC class I is associated with higher embryo cleavage rates, faster cell division and increased blastomere numbers, in humans and mice. However the

precise role of these molecules in preimplantation human embryos remains elusive, and the ability to manipulate and/or measure their expression in order to improve IVF success rates remains problematic. Commercial dairy cattle experience high levels of early embryo loss. They are monovular, non-seasonal breeders with a similar gestation period to humans, and they also share many immune system components. Understanding the role of MHC expression in preimplantation cattle embryos can thus benefit the dairy industry and contribute to human health. We can access and manipulate large numbers of cattle embryos, and are in the unique position of having a range of MHC-homozygous animals, and can thus create embryos with precise combinations of classical and non-classical MHC genes. Expression studies revealed that in term cattle placenta classical MHC class I is absent or moderate, while non-classical class I transcription has been detected in third trimester trophoblast tissue, and binucleate trophoblast cells. The objective of our current study is to characterize mRNA and protein expression profiles of MHC class I genes during early preimplantation development in cattle embryos. Findings thus far reveal that transcripts for both classical and non-classical MHC class I genes are present during development from the immature oocyte until at least the hatched blastocyst stage.

MS2-4

Current aspects on the involvement of HLA and KIR genes in abortion

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The interactions between receptors of the KIR family and specific HLA-C molecules expressed on trophoblast appear to be involved in the function of an NK cell mediated pregnancy allorecognition system. Given the differences in both the KIR repertoire and the HLA-C allotypes among unrelated individuals, each pregnancy presents a different combination of maternal inhKIR receptors on decidual NK cells (dNK) and trophoblastic self and non-self HLA-C allotypes. This combination is expected to ensure the appropriate receptor–ligand interactions to inhibit dNK anti-trophoblast activity, thus to favor

pregnancy. In contrast, abortion may be the result of a defect in this NK allorecognition system. Studies in RSA couples as well as in random cases of abortion have suggested that aborting women have a limited repertoire of inhKIR receptors and that in many cases there is a lack of maternal inhKIR-fetal HLA-C epitope matching. However, this suggestion has not been confirmed and it seems more likely that the abortogenic effect depends on the combination of activating and inhibitory KIRs on aborters’ KIR haplotypes. There is evidence that in women with RSA of alloimmune aetiology the ratio of inhibitory to activating KIRs is lower than that in fertile women and those with autoimmune abortions, because they possess a rather uncommon KIR gene repertoire where A KIR haplotypes contain extra activating KIRs. It is possible that this imbalance in favour of activating KIRs gives to these women a high potential for dNK cell activation, which, if not controlled by the limited inhKIR-HLA-C interactions, may damage trophoblast and avert pregnancy.

MS2-5

Placenta-derived soluble MHC class I chain-related molecules MICA and MICB down-regulate the NKG2D receptor on the maternal peripheral blood mononuclear cells: a novel immune escape mechanism for fetal survival

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Problem: The acceptance of the fetus by the maternal immune system is dependent on the placenta that is not only the source of nourishment and pregnancy hormones but also an important immunoregulatory organ. In search of molecular mechanisms responsible for the maternal tolerance to the fetus we have chosen to analyse the most powerful and thus most dangerous for the fetus cytotoxic immune mechanism based on the interactions of the activating killer receptor NKG2D and its ligands MICA and MICB (MIC) during pregnancy.

Method of study: Early and term placenta, PBMC and pregnancy sera were investigated for NKG2D/MIC receptor/ligand expression and interplay using

quantitative RT-PCR, immunohistochemistry, ELISA and cytotoxic assays.

Results: MIC mRNA was constitutively expressed by human placenta and upregulated upon heat shock treatment. MIC expression was restricted to the apical and basal membrane of the syncytiotrophoblast, and to cytoplasmic vacuoles as MIC-loaded exosomes. Soluble MIC (sMIC) were present at elevated levels in maternal blood throughout normal pregnancy. Simultaneously, the NKG2D expression on maternal PBMC was downregulated compared to non-pregnant controls. sMIC molecules in pregnancy serum were able to downregulate the NKG2D recep-

tor on PBMC from healthy donors, with the consequent inhibition of the NKG2D-dependent cytotoxic response.

Conclusions: A mechanism for fetal evasion of maternal immune attack, based on the engagement and downregulation of NKG2D on PBMC by sMIC molecules is described. These findings suggest a novel physiological mechanism of silencing the maternal immune system, which promotes fetal allograft survival and supports the view of placenta as an immunoregulatory organ.

MAIN SESSION 3: IMMUNO-ENDOCRINE INTERACTIONS IN REPRODUCTION

MS3-1

Cytokine expression at interface: a longitudinal micro arrayG Chaouat¹, N Coqué³, B Hennuy⁴, S Dubanchet¹, I Bataillon², N Rodde², N Ledée-Bataille², O Sandra²¹INSERM U782 Clamart; ²INRA Jouy en Josas; ³INAPG Paris (France);⁴Tumor & Development Laboratory CHU University of Liège (Belgium)

Problem: A complex cytokine network is expressed in a time dependent fashion in the pre and peri implantation uterus, whose knowledge is still limited. Preliminary microarrays have already been conducted showing the feasibility and limits of the technique

Method: Sequential day by day micro array on full expression in CBA x DBA/2 or BALB/c murine combinations starting day -1.

Results: Data will be shown and the main results summarised; Important daily variations are seen in early steps

Conclusion: As already pointed out by some, events are sequential and there is no "fixed expression profile" in the pre and peri implantation period. The presence of the embryo seems to indicate the need for laser micro dissection studies.

MS3-2

Factors responsible for the cytokine profile of T cells in pregnancy

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Problem: In women suffering from unexplained recurrent abortion a defect of the IL-4, IL-10, leukemia inhibitory factor (LIF) and M-CSF production by decidual CD4+ T cells in comparison with decidual T cells cytokine production of fertile women undergoing a voluntary abortion, has been shown (Piccinni MP et al. Nat Med 1998; 4:1020–1024). The reduced production of these cytokines was not

seen at the level of peripheral blood, suggesting that this is not an inherent feature of T cells, but rather a microenvironmentally oriented alteration.

Method of study: The role of hormones (progesterone, relaxin, hCG, 17 β estradiol, analogues of progesterone) and molecules expressed and/or produced by trophoblast and by uterine stromal cells were examined for their capacity to modulate the decidual T cell cytokine profile during pregnancy.

Results: Progesterone is a potent inducer of IL-4, LIF and M-CSF production by T cells. Soluble HLA-G1, a class I molecule secreted by trophoblast, and molecules expressed on the membrane of uterine stromal cells were able to increase the decidual T cell-IL-4 production, whereas molecules expressed on the membrane of trophoblast cells were able to increase IL-10 production by uterine T cells during pregnancy.

Conclusions: Many factors present in microenvironment of T cells in the decidua could be responsible for the decidual T cell pattern.

MS3-3

PIBF signaling in decidual cellsJ Szekeres-Bartho¹, A Balogh¹, M Halasz¹, N Kozma¹, U Markert², E Miko¹, T Palkovics¹, B Polgar¹, T Poehlmann²¹Department of Medical Microbiology and Immunology MedicalSchool, Pecs University, Pecs, Hungary; ²Friedrich Schiller University, Jena, Germany

Problem: PIBF acts on the cytokine pattern, which contributes to its anti-abortive effect in mice. We aimed to investigate PIBF signaling in peripheral mononuclear cells and decidual lymphocytes.

Method of study: Proteins from lysates of PIBF-treated peripheral mononuclear cells as well as those of untreated control cells were reacted on Western blots with antibodies recognizing phosphorylated STATs, Jaks and SOCS molecules. STAT6 phosphorylation in decidual cells was detected by flow cytometry. Nuclear translocation of STAT molecules was detected with EMSA.

Results: PIBF activates STAT6 and inhibits the phosphorylation of STAT4. Activation of the STAT6

pathway depends on ligation of the IL-4 receptor, thus it seems plausible that the IL-4R is involved in PIBF-induced STAT6 activation. Though PIBF does not bind to IL-4R, blocking of the latter abolishes PIBF-induced STAT6 activation, whereas blocking of the IL-13 receptor has no effect. Furthermore, PIBF fails to signal in IL-4R knock down cells. The requirement of the IL-4R for PIBF signaling could be due to the lack of intracellular domains in the PIBF receptor, therefore the possibility of the PIBF receptor being a GPI anchored protein was considered. Digesting the GPI-anchor abolished the STAT6 activating effect of PIBF, while that of IL-4 remained intact.

Conclusions: These data suggest the existence of a novel type of IL-4R, composed of the IL-4R and the GPI-anchored PIBF receptor. Upon ligation the GPI-anchored PIBF receptor combines with the alpha chain of the IL-4 receptor, and induces Jak1 phosphorylation, which in turn activates STAT6.

MS3-4

Progesterone-dependent signaling in uterine biology and implantation

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Progesterone is one of the key players in interaction between endocrine and immune systems in the uterus that regulates menstrual bleeding, tissue repair and regeneration, inflammation, angiogenesis, blastocyst implantation, and maintenance of pregnancy. The progesterone responsiveness is determined by the extent of expression of specific progesterone receptors which mediate progesterone action via genomic and non-genomic pathways. In human, rodents, and many other species the genomic actions of progesterone are mediated by intracellular receptors, progesterone receptor A (PR-A) and progesterone receptor B (PR-B), which are members of the nuclear receptor superfamily of transcription factors, and are the product of the same gene transcribed under control of two distinct promoters. The receptors PR-A and PR-B are phosphoproteins with transcriptional activity dependent on the state of phosphorylation of the cognate receptors and/or their coregulator proteins. Spatial and temporal expression of the PR-A and PR-B vary in reproductive tissues as a consequence of the

developmental and the hormonal status and during carcinogenesis. The non-genomic action of progesterone is characterized by a rapid response to progesterone (latency of minutes rather than hours), which is independent of transcription regulatory functions of PRs and is executed by at least two mechanisms. The first is PRs mediated via activation of the intracellular phosphorylation cascades. The second mechanism is PR-A and PR-B independent and appears to operate through membrane specific receptors for progesterone. Proper functional communication between the genomic and non-genomic progesterone regulated signaling pathways could be of critical importance for the correct endocrine-immune interaction in uterus during the establishment and the maintenance of pregnancy. Current studies of the progesterone regulated signaling pathways and downstream targets of progesterone will be discussed.

Supported by European FVI project (EMBIK no 512040) and Croatian Ministry of Science, Education and Sport (no 062-0620402-0381) grant.

MS3-5

Pathomechanism of testicular inflammation

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There is now widespread agreement that the immune system, spermatogenesis and steroidogenesis, the intrinsic testicular functions, are intricately linked by a network of complex interactions. The importance of the delicate balance needed between the suppression of the immune response to protect the germ cells from autoattack on the one hand, and the ability to an active immune response to prevent damage from infection, trauma and cancer on the other, is reflected by the fact that in the human male about 12-13%, in some studies even more, of all diagnosed infertility is related to an immunological reason, whilst its contribution to idiopathic infertility (31% of all cases) remains elusive. Autoimmunity, in the form of autoantibodies, symptomatic and non-symptomatic inflammation of the testis (orchitis) due to reproductive tract infections all lead to the disruption of testicular androgen production and spermatogenesis. As some of these conditions are associated with almost

indistinguishable clinical symptoms, the diagnosis of chronic, "silent" infection/inflammation of the epididymis and testis is difficult and usually overlooked. Orchitis can be caused by traumatic events as well as bacterial and viral infection. In men, it is usually associated with epididymitis. The most common cause is mumps orchitis, but orchitis also occurs in conjunction with bacterial infections of the male genitourinary tract and *E. coli* is the most frequent pathogen isolated in semen. This lecture will focus on the identification of high-affinity testicular autoantibodies targeted in orchitis and the mechanism how *E. coli* strains are able to suppress testicular immune defense during infection.

MS3-6

Local and systemic regulation of uterine lymphocytes and macrophages in ruminants

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The number, type, and function of immune cells in utero are regulated during pregnancy to allow for fetal survival. In sheep, for example, pregnancy is accompanied by an increase in gamma-delta T cells in luminal epithelium, a decrease in other T cells in epithelium, and an increase in macrophages in stroma. Experiments using unilaterally-pregnant sheep, in which the conceptus is confined to one uterine horn, indicate that these changes are driven by local signals from the conceptus and hormonal changes mediated by the placenta or maternal system. Knowledge is incomplete regarding specific molecules that regulate uterine immune function. The most well-characterized molecule is progesterone which can block tissue graft rejection in utero in sheep. Progesterone probably inhibits uterine immune responses indirectly by inducing secretion of a member of the serine proteinase inhibitor family called uterine serpin from the endometrial epithelium. Uterine serpin, which is present in sheep, cattle and pigs, can block lymphocyte proliferation *in vitro* in sheep and natural killer cell mediated abortion *in vivo* in mice. In the cow, transcripts for non-classical MHC antigens have been identified in the placenta; these proteins could be involved in regulation of antigen-presenting cells or other immune cells. The cow undergoes a period of

systemic immunosuppression around parturition and it is possible that regulatory T cells are involved. During late pregnancy, there is an increase in the proportion of peripheral blood lymphocytes that are CD4⁺CD25⁺. Thus, changes in immune function to allow pregnancy may compromise immune function in the periparturient period.

MS3-7

Immunology of endometriosis

A Arici

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Endometriosis is classically described as the presence of both endometrial glandular and stromal cells outside the uterine cavity, mainly in the pelvis. The pathogenesis of this enigmatic disorder still remains controversial despite extensive research. Although multiple theories have been put forth to explain the pathophysiology and pathogenesis of endometriosis, the retrograde menstruation theory of Sampson is the most widely accepted. However, since retrograde menstruation occurs in most of the reproductive age women, it is clear that there must be other factors which may contribute to the implantation of endometrial cells and their subsequent development into endometriotic disease. Endometriosis is associated with an inflammatory peritoneal environment, where multiple cytokines and growth factors are found at elevated levels. There is substantial evidence to support that the alterations in both cell-mediated and humoral immunity contribute to the pathogenesis of endometriosis. Increased number and activation of peritoneal macrophages, and decreased T cell and NK cell cytotoxicities are the alterations in cellular immunity and result in inadequate removal of ectopic endometrial cells from the peritoneal cavity. Moreover, increased levels of several cytokines (i.e., interleukin-8) and growth factors which are secreted by either immune or endometrial cells seem to promote implantation and growth of ectopic endometrium by inducing proliferation and angiogenesis. In addition to the impaired capacity of the immune cells to mediate endometrial cell removal, inherent resistance of the ectopic endometrial cells against immune cells is another interesting concept in the pathogenesis of endometriosis.

MAIN SESSION 4: CYTOKINES AND SIGNALING AT THE MATERNAL FETAL INTERFACE

MS4-1

A role of IL-18 in expansion of distinct lymphocyte populations

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IL-18 was originally discovered as an IFN- γ -inducing factor and is regarded as a pro-inflammatory cytokine. However, recent studies revealed that IL-18 is a pleiotropic, paradoxical cytokine that enhances both Th1 type and Th2 type cytokines dependently on co-existing factors, augments cytolytic activity of NK cells and CTL, and up-regulates adhesion molecules. Thus, it is necessary to elucidate the principle of biological action of IL-18 in order to understand the roles of IL-18 in inflammation. Here I emphasize that IL-18 promotes survival and expansion of some subsets of activated lymphocytes. Proliferation and survival of mouse CD8⁺ T cells, stimulated by immobilized anti-CD3 antibody, was strongly impaired by IL-18 deficiency, which was reversed by the addition of IL-18. Expression of IL-18Rs, particularly IL-18R β chain, in naïve CD8⁺ T cells was very low, but elevated after stimulation. IL-18 deficiency altered the expression of various surface markers such as CD28, CD94, CD122, and NKG2A, which may be related to cell viability and function. Levels of phosphorylated Akt and Bcl-2 in IL-18-deficient CD8⁺ T cells were lower than those in WT cells, but were raised by exogenous IL-18. In cultures of human PBMC, IL-18 markedly promoted expansion of several subsets of cells including NK cells. IL-18 activates NF- κ B, a cytokine-inducing signal, and in the downstream of NF- κ B, molecules involved in cell survival, such as cIAP and XIAP were activated. I hypothesize that IL-18 plays regulatory roles in immune/inflammatory responses by influencing population size of functional subsets through its novel role as a cell survival promoting factor in terminally differentiating cells.

MS4-2

The balance between immunostimulation and immunoregulation during pregnancyS Saito, A Nakashima, M Ito, T Shima, A Shiozaki
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Problem: We discuss the role of regulatory T (Treg) cells which induce MHC class II specific tolerance and regulatory NK (NKreg) cells which induce MHC class I specific tolerance in the maintenance of pregnancy.

Method of study: Peripheral blood or decidual sample was collected from the normal pregnant subjects or miscarriage cases. Lymphocyte subsets and cytokine profile were analyzed by flow cytometry.

Results: Peripheral blood CD4⁺CD25⁺Foxp3⁺ Treg cells increased from early pregnancy. The frequencies of Treg cells in the decidua were significantly higher than those in peripheral blood. Decidual Treg cells expressed surface CTLA-4 suggesting that Treg cells recognize some antigens such as fetal antigens. And decidual CD4⁺CD25⁺ Treg cells inhibited the T cell proliferation by cell-to-cell interaction. This CTLA-4 molecule induced IFN- γ production by decidual dendritic cells (DCs) and macrophage (M ϕ), and induced IDO enzyme. On the other hand, decidual Treg cells decreased to non-pregnancy level in miscarriage case and IDO expression in DC and M ϕ also decreased in miscarriage cases. The cytokine profile in decidual and peripheral blood NK cells were unique. TGF- β producing NK3 cells increased in early pregnancy decidua and IL-10 producing NKr1 cells increased in peripheral blood of normal pregnant women. In vitro data showed that soluble HLA-G1 and prolactin induced TGF- β production by NK cells, and HCG and progesterone induced IL-10 production by NK cells. On the other hand, these cytokine profiles in miscarriage cases change to non-pregnancy state.

Conclusions: Treg cells and NKreg cells closely cooperate on the tolerance induction during pregnancy.

MS4-3

Crucial roles for Wnt signaling in Implantation

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One of the crucial events during mammalian embryogenesis is the process of implantation. Successful implantation depends on precisely orchestrated and reciprocal signaling between the implanting blastocyst and the receptive uterus. We have been investigating the role of the Wnt signaling as a potential pathway involved in embryo-uterine cross talk. We have shown that the blastocyst, prior to implantation, expresses multiple Wnt genes, and that the Wnt/ β -catenin signaling pathway is activated in the uterus by the blastocyst at the future site of embryo attachment. Activation of this pathway is required for implantation. Furthermore, we have demonstrated that estrogen as well as leukemia inhibitory factor (LIF) signaling in the uterus is required prior to implantation for activation of the Wnt pathway in the luminal epithelium of the uterus by the blastocyst. We have demonstrated that Wnt signaling components show a dynamic pattern of expression during the peri-implantation stages and several genes require LIF signaling for proper expression. This differential expression pattern highlights a possible mechanism of regulation of the Wnt pathway in the uterus, where LIF modulates the expression of component of Wnt signaling.

MS4-4

Stress during pregnancy: consequences for mother and child

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Psychological stress is a prevailing facet of daily life and high stress perception perceived by women during their reproductive years has long been suspected as a possible cause of infertility, implantation failure, late pregnancy complications and impaired fetal development. Apart from the classical stress-related neurohormones like the players of the hypothalamus-pituitary-adrenal (HPA) axis or catecholamines neurohormones and neurotrophins, it is well known

that stress exposure decreases levels of luteinising hormone and progesterone in mammalian pregnancies, which profoundly challenges immune tolerance towards the fetus. Besides pregnancy complications such as spontaneous miscarriages, compelling evidence from epidemiological studies and animal research further indicates that offspring who experience stress in utero are more likely to develop chronic health problems as adults, such as heart disease, high blood pressure, diabetes and atopic diseases. In this context, it has been hypothesized that the effects of maternal psychosocial stress and stress-related maternal endocrine dysregulation affects fetal developmental and health outcomes. Endocrine-immune dysregulations during pregnancy in response to psychological stress are becoming increasingly defined, e.g. due to the development of particularly instructive rodent models. Such insights may then prompt clinicians to become far more attentive to the effect of psychological stress on pregnancy complications. Together with basic science research elucidating hierarchical, temporal and spatial interactions of key endocrine and immune parameters at the fetomaternal interface during central and peripheral responses to psychological stress, a list of candidate molecular targets for clinically useful therapeutic intervention of pregnancy complications can then be created.

MS4-5

The STAT3/SOCS3 balance and trophoblast invasion

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Several papers have indicated a leading role of Signal Transducer and Activator of Transcription 3 (STAT3) in induction of invasiveness of tumors. This has been confirmed also for choriocarcinoma and trophoblast cells. STAT3 can be phosphorylated by several growth factors and cytokines, mainly of the IL-6 family. STAT3 exists in two isoforms: STAT3-a has a serine (ser727) and a tyrosine (tyr705) phosphorylation site, while the splicing variant STAT3-b has only tyr705. The role of both is still under investigation. Phosphorylated STATs form dimers which induce transcription of a variety of proteins. These

include Suppressor of Cytokine Signalling 3 (SOCS3) which is a major negative regulator of STAT3 and, thereby, regulator of invasiveness. Current works indicate that the balances of serine and tyrosine

phosphorylated STAT3 and SOCS3 may be key regulators of trophoblast invasion.

MAIN SESSION 5: MUCOSAL IMMUNOLOGY OF THE FEMALE REPRODUCTIVE TRACT

MS5-1

Induction of mucosal immunityER Podack¹, N Strbo¹, S Pahwa¹, M Kolber¹,
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Fusion protein gp96-Ig retains its function as primary endoplasmic reticulum-chaperone for peptides and proteins, but is secreted from cells expressing it due to the replacement of the KDEL retention signal with the IgG1 Fc-tag. Tumor cells secreting gp96-Ig are highly immunogenic and induce CD8 CTL responses against tumor antigens even in the absence of CD4 cells, CD40-L and lymph nodes. Gp96-chaperoned peptides are 10 million fold more efficiently cross presented by antigen presenting cells to CD8 cells than the corresponding intact protein. We found that cell based vaccines secreting gp96-Ig are more efficient in generating CD8 CTL responses than equivalent amounts of purified gp96-Ig isolated from cell culture supernatants. We suggest that continuous *in vivo* secretion of gp96-Ig from the vaccine cell may mimic the immunizing effect of attenuated viruses with limited replication potential. Many infections, including HIV, are transmitted through mucosal surfaces. Protective and therapeutic immunity therefore requires vaccines generating strong mucosal immune responses. Measuring antigen specific CD8 CTL responses, we show that gp96-Ig secreting cells injected intraperitoneally in addition to generating systemic immunity, stimulate expansion and migration of CD8 CTL to mucosal sites including the intraepithelial compartment (IEL), the lamina propria (LPL) and Peyer's patches (PPL). CCR9, $\alpha 4\beta 7$ and $\alpha E\beta 7$ (CD103) expression on activated CD8 cells in the mucosal compartments indicates that priming by gp96-Ig induces the appropriate trafficking and homing markers. Intraperitoneal immunization thus induced both, strong mucosal and systemic immunity. Intradermal immunization was unable to induce CD8+ IEL but generated moderate responses in the lamina propria and Peyer's patches. Subcutaneous immunization with gp96-Ig secreting cells generated both systemic and mucosal responses, but with less intensity than

i.p.-immunization. Gp96-Ig secreting cells were instilled intranasally, intravaginally and intrarectally in order to determine whether the application of gp96-Ig-secreting cells directly to mucosal epithelia generated mucosal and/or systemic CD8 CTL responses. Rectal and vaginal instillation of gp96-Ig secreting cells induced strong, antigen specific mucosal CD8 responses (IEL, LPL, PPL) in the complete absence of systemic responses (spleen, peripheral lymph nodes). The data indicate that cell-secreted gp96-Ig is a highly efficient vaccine for CD8 CTL induction. Generation of immune responses at different sites can be controlled by choosing appropriate vaccination routes. Continuous cell mediated secretion of gp96 is more effective than bolus gp96 injection. Gp96-chaperoned peptides are more than million-fold more effective in CD8 CTL cross priming than intact protein.

MS5-2

Innate immunity in the human female reproductive tract (FRT): Role of estradiol in innate protection by mucosal epithelial cells

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Problem: Uterine epithelial cells (UEC) are sentinels of the innate immune system, expressing proinflammatory cytokines and chemokines and anti-microbial molecules. This study was undertaken to determine how estradiol (E_2) influences human uterine epithelial cell protection.

Method of study: FRT epithelial cells were isolated and cultured in cell inserts to confluence and high transepithelial resistance (TER), prior to treatment with E_2 and/or the TLR3 agonist poly (I:C). The secretion of cytokines and chemokines were examined as well as the mRNA expression of antimicrobials and the IFN β -stimulated genes myxovirus resistance gene 1 (Mx1) and 2',5' oligoadenylate synthetase (OAS).

Results: Poly (I:C) induced the secretion/expression of cytokines TNF α , IL-6, GM-CSF, and G-CSF, chemokines IL-8, MCP-1 and MIP-1 β as well as antimicrobials HBD1, HBD2 and MIP3 α . Poly (I:C), treatment of UEC initiated an antiviral response by inducing IFN β , Mx1 and OAS mRNA expression. In other studies, E₂ pre-treatment blocked the secretion of MIF, IL-6 and GM-CSF induced by LPS and poly (I:C). In contrast, E₂ increased the secretion/expression of the microbicides/chemokines HBD2 and SLPI, and with poly (I:C) synergistically enhanced MIP3 α mRNA expression.

Conclusions: These results indicate that UEC respond to TLR agonists by producing proinflammatory cytokines and chemokines that mediate the initiation of inflammatory responses. They also express antimicrobials and IFN β -stimulated genes that can have a direct inhibitory effect on microbe replication. Further, these studies indicate that E₂ can selectively inhibit the production of pro-inflammatory cytokines, while enhancing factors that are microbicidal for pathogens. Supported by AI51877 from NIH.

MS5-3

Estrogen receptor ligands regulate dendritic cell differentiation

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Problem: Studies of immune responses in the normal state, during autoimmunity and after infection or trauma indicate that estrogen receptor (ER) ligands can modulate innate and adaptive immunity and hematopoiesis. We have found that ER signaling differentially regulates dendritic cell (DC) differentiation mediated by GM-CSF and Flt3 Ligand (FL). While FL is most important for constitutive DC development, elevated GM-CSF during inflammation and autoimmunity may increase DC differentiation. Our goal is to define the DC progenitors in murine bone marrow that respond directly to estradiol and to determine the molecular mechanisms by which ER signaling regulates DC differentiation. We also are investigating a role for ER ligands during DC differentiation *in vivo*.

Method of study: The effects of ER ligands (estradiol, the ER antagonist ICI 182,780, tamoxifen) on murine DC differentiation from ER α ⁺ bone marrow myeloid progenitors are being studied in cytokine-driven culture models and after imposition of tamoxifen *in vivo*.

Results: In GM-CSF supplemented cultures initiated from myeloid progenitors, estradiol promoted differentiation of functional DC with features of Langerhans cells. Conversely, in FL supplemented cultures initiated from myeloid progenitors, estradiol inhibited cell survival, thereby decreasing the yield of plasmacytoid and myeloid DC. Experiments with bone marrow cells from ER α ^{-/-} or ER β ^{-/-} mice showed that estradiol acted via ER α to regulate DC differentiation.

Conclusions: Our data show that estradiol, acting primarily via ER α , exerts opposing effects on the same myeloid progenitor during GM-CSF or FL mediated DC differentiation, suggesting that ER signaling in DC progenitors alters cell fate by differential synergy with cytokine receptor signals.

MS5-4

Effect of local microenvironment on susceptibility and immune responses to sexually transmitted viruses in the female genital tract

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Problem: Sexually transmitted viruses, HIV and HSV-2, are a major health problem and a huge burden on health systems, globally. Not much is understood about pathogenesis of these viruses in the local environment of the female reproductive tract, even though majority of infections occur heterosexually and women carry the major burden of infection. We believe that the initial interaction of viruses with the genital tract determines the quality of immune response and subsequent outcome of infection. Further, the local environment in the genital mucosa plays a critical role in determining the quality of immune responses to infection.

Methods: We have developed *in vivo* and ex-vivo viral infection models that we are using to examine

the interactions of HIV-1 and HSV-2 with the female genital tract.

Results: Using a mouse model of genital HSV-2 infection, we have shown that reproductive hormones, estradiol and progesterone, have a profound effect on the outcome of infection and the quality of immune response. We have also developed an ex-vivo culture system that allows us to directly examine the interaction of sexually transmitted viruses with female genital tract cells. Using this system we are developing novel approaches to prevent the initiation of infection as well as augment the innate immune system to protect against STIs.

Conclusions: Clear understanding of local events, and factors that influence them within the genital tract, will allow us to subsequently manipulate local immune responses. This has important implication both for designing preventative strategies, vaccine formulations, as well as for women who are currently using hormonal treatments or contraceptives.

MS5-5

Who stands on guard for thee? Innate protection against sexually transmitted infections in mice and humans

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Problem: Although activation of Toll-like receptors induces potent anti-microbial protective effects, incomplete understanding of the mechanisms involved limits clinical utility.

Method of study: Female wild-type and bone-marrow (BM) chimeric mice which expressed TLR9 on either epithelial/stromal cells or BM-derived cells or both were studied. Mice were treated intravaginally (IVAG) with CpG DNA and protection assessed against genital HSV-2 infection. The phenotype of cells secreting type I interferons (IFN) was determined using FACS and intracellular cytokine staining.

Results: We describe novel and unique vaginal interferon-producing cells (vIPCs), which are distinct from plasmacytoid dendritic cells (pDCs), and responsible for the majority of type I interferon production in vaginal tissue. Phenotypically, in addition to expressing CD11c and Gr-1, they also expressed CD11b and F4/80, but were largely negative for PDCA-1 and B220. Furthermore, vIPCs differed from pDCs in that they produced large amounts of IFN- β , and little to no IFN- α . IVAG delivery of CpG DNA rapidly penetrated and disseminated throughout the vaginal submucosa and expanded vIPCs. Importantly, epithelial and stromal cells were shown to play a marginal role in the production of type I IFN, and are dispensable for the IVAG CpG-mediated innate anti-viral state. Lastly, depletion of vIPCs abrogates the protective effects of CpG against genital HSV-2 infection.

Conclusions: These results demonstrate that vIPCs represent a unique cellular subset, the stimulation of which is both necessary and sufficient for the induction of the CpG-mediated innate anti-viral state. Improved innate antiviral protection may result from more efficient targeting of vIPCs in the genital tract.

MAIN SESSION 6: HIV AND REPRODUCTION

MS6-1

Mucosal immunology of HIV-1 infections**J Mestecky, Z Moldoveanu, S Jackson, PA Goepfert**

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Infection with HIV-1 must be primarily considered as a mucosal disease: the absolute majority of infections is acquired through the genital and intestinal tracts, a high level of virus replication takes place in the lymphoid compartment of the gut, and the earliest and most dramatic immunological alterations (depletion of CD4⁺ T cells) occur in mucosal tissues. Considering the pivotal role of immunoregulatory CD4⁺ T cells, their depletion compromises mucosal and systemic defenses. Evaluations in our laboratory of humoral immune responses in sera and external secretions (tears, saliva, milk, urine, semen, and nasal, vaginal and rectal washes) from HIV-1-infected, HIV-1-exposed but seronegative, or HIV-1 vaccine immunized individuals revealed several unexpected findings. Although the levels of total IgG and IgA were elevated or unchanged in all body fluids, the dominant isotype of HIV-1 specific antibodies was IgG in all fluids examined, despite the dominance (~95%) of total IgA in saliva, intestinal fluid, and milk. HIV-1-specific antibody-secreting cells in peripheral blood were dominantly of the IgG isotype, irrespective of the route of infection, CD4⁺ T cell counts and virus loads in peripheral blood, and the duration of infection. In contrast to other reports, we did not find HIV-1-specific IgA antibodies in sera and external secretions of HIV-1-exposed but seronegative individuals. Finally, in humans, mucosal or systemic immunizations with experimental HIV-1 vaccines did not induce HIV-1-specific antibodies and antibody-secreting cells of the IgA isotype. Whether humoral responses to previously or newly encountered non HIV-1-related antigens are ablated in HIV-1-infected individuals is under investigation.

MS6-2

HIV-specific CD8+ T-cell responses in mucosal tissues**JW Critchfield¹, D Lemongello¹, TL Hayes¹, AL Ferre¹, DH Young¹, JC Garcia³, RB Pollard^{1,2}, BL Shacklett^{1,2}**¹Department of Medical Microbiology and Immunology; ²Department of Internal Medicine, Division of Infectious Diseases; ³Division of Gastroenterology, University of California, Davis, CA, 95616, USA

Problem: Mucosal tissues are an important site of HIV replication and CD4 depletion. Previous studies demonstrated that mucosal CD8 T-cells differ in phenotype and function from their counterparts in blood. We assessed HIV-specific T-cells in blood, rectal and cervical mucosa for antigenic breadth and production of effector molecules (IFN γ , TNF α , IL-2, MIP-1 β , CD107).

Method of Study: Blood and rectal biopsy tissue were obtained from 35 HIV+ individuals and 7 controls; cervical lavage and/or cytobrush specimens were obtained from 15 HIV+ women and 3 controls. HIV-specific T-cell functions were assessed by intracellular cytokine staining using 10-color flow cytometry. Positive responses were defined as >0.05% of CD8+ T-cells after subtracting controls. For epitope mapping, cells were expanded and tested in an IFN(Elispot with peptides corresponding to HIV-1 Gag, Env and Nef. Positive responses were defined as >50 SFC/10⁶ after subtracting background.

Results: Mucosal CD8+ T-cells displayed a complex pattern of functions. HIV-specific responses fell into distinct categories which differed between CD4+ and CD8+ T-cells and between blood and mucosal tissues. The magnitude and breadth of Gag-specific responses were generally greater in rectum than blood. The number of viable cells obtained from cervical cytobrush specimens was limited; however, preliminary comparisons of HIV-specific T-cell responses in rectal and cervical mucosa suggested overlap in the fine specificity of T-cells at these two very different anatomical sites.

Conclusions: Mucosal CD8+ T-cells are capable of broad and complex HIV-specific responses, and likely play a major role in immune surveillance of these tissues.

MS6-3

A *Plasmodium falciparum* Antigen Increases HIV-1 Replication in a Human Placental Derived Cell Line

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Problem: Epidemiological data have established a link between placental malaria and an increased risk for HIV-1 mother-to-child transmission (MTCT), by unknown mechanisms.

Methods: The placenta-derived cell line BeWo and monocyte-derived macrophages (MDM) were infected with varying doses of luciferase reporter HIV-1 pseudotyped with the vesicular stomatitis virus protein G. A recombinant DBL3 γ domain, derived from the adhesin PfEMP1 of a *Plasmodium falciparum* parasite line collected from an infected placenta (DBL3 γ -732), that binds to chondroitin sulfate A (CSA), was used to stimulate infected cells. In some experiments, DBL3 γ -732 was preincubated with the Fab fragment of a specific monoclonal antibody that inhibits binding to CSA. TNF- α was measured in the culture supernatants and luciferase activity was quantified at different time points in cell lysates to evaluate viral replication.

Results: Addition of DBL3 γ -732 to HIV-1-infected BeWo cells led to a dose-dependent increase of HIV-1 replication. This enhancement was specific as it could be inhibited by the Fab fragment of a monoclonal antibody against DBL3 γ -732. In MDM, by contrast, the addition of the same concentrations of DBL3 γ -732 induced a strong dose-dependent inhibition of HIV-1 replication. The effect of DBL3 γ -732 on HIV-1 replication is most likely mediated by TNF- α as this cytokine was increased by DBL3 γ -732 binding to BeWo cells and MDM.

Conclusions: This study identified for the first time a direct link between a specific *Plasmodium* antigen and HIV-1 replication. These data underline the

importance of efficient malaria prophylaxis together with antiretroviral interventions in areas where HIV-1 and malaria co-circulate.

MS6-4

The pros and cons of dendritic cells in HIV transmission

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Problem: Being positioned within the tissues lining the body, dendritic cells (DCs) are central to innate and adaptive immunity to incoming pathogens. HIV exploits DC biology to facilitate the initiation and spread of HIV infection. A better understanding of how HIV crosses the reproductive epithelia (in the presence and absence of other infections like HSV-2 that exacerbate HIV spread) is needed to improve preventative vaccines and microbicides.

Method of study: Model monocyte-derived and skin-derived DCs are studied for their abilities to interact with HIV and HSV-2. DC biology is monitored by flow cytometry and by measuring cytokine/chemokine release. Infections are monitored by PCR, ELISA, and immunostaining. In parallel, we perform studies in macaques to evaluate this biology *in vivo*.

Results: HIV does not fully activate DCs like many other pathogens do, resulting in suboptimal immunity being induced. DCs readily capture HIV, which can be internalized and rapidly transferred to CD4 T cells. Immature DCs can also become infected by HIV and then transfer the newly produced viruses to CD4 T cells. HSV-2 can infect immature macaque DCs and impair their immunostimulatory activity. Vaginal challenge of macaques with HSV-2 coincides with the rapid appearance of lesions (primarily cervical) and heightened cytokine and chemokine responses. How this impacts immunodeficiency virus is under investigation.

Conclusions: HIV and HSV-2 subvert DC biology in very different ways that likely act together to favor HIV spread. Identifying ways to circumvent virus-induced dysfunction is key to limiting HIV infection.

MAIN SESSION 7: NK CELLS IN IMPLANTATION AND PREGNANCY

MS7-1

Tolerance and natural killer cells

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Problem: Natural killer (NK) cells are found at the maternal-fetal interface. Relevant to full appreciation of uterine NK cells is how NK cells achieve self-tolerance because their potent effector capacities must be controlled to avoid inadvertent destruction of otherwise normal tissues. Through their inhibitory receptors, NK cells can be regulated by MHC class I molecules on their cellular targets. However, MHC class I-deficient hosts do not have overactive NK cells, and instead their NK cells have poor target killing capacity. Furthermore, the NK cell receptors are not uniformly expressed and are encoded by polymorphic genes that segregate independently from genes for their highly polymorphic ligands, suggesting that each NK cell must acquire self-reactivity.

Method of study: We used a deceptively simple cell-free assay to determine the functional competence of freshly isolated NK cells to be triggered through their activation receptors.

Results: MHC class I-specific receptors for self-MHC impart signals allow NK cells to acquire the functional capacity to be triggered by their activation receptors, a process termed "licensing." Ironically, the self-MHC-specific receptors were originally identified in terms of inhibition of NK cell effector responses.

Conclusions: These studies strongly suggest that the MHC-specific receptors directly engage self-MHC class I in order to generate two types of self-tolerant NK cells, i.e., licensed NK cells that are inhibited by self-MHC through the same receptor that conferred licensing, and unlicensed NK cells that do not need to be inhibited by self-MHC since they are unlicensed.

MS7-2

Molecular mechanisms involved in the control of NK cell accumulation in the decidua

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During early pregnancy, decidualization of the human uterine mucosa is accompanied by a drastical enrichment of CD56^{high}CD16^{negative} NK cells which represent the most prominent immune cells in the first half of human pregnancy. Uterine NK (uNK) cells differ from CD56^{high} blood NK cells in several ways, including the KIR receptor repertoire, the adhesion molecule and the chemokine receptor profile as well as for the expression of some genes induced by the hormone environment. However, it is still unclear whether uNK cells arise from an NK cell progenitors present in the uterus or recruited from other tissues or peripheral blood that once in the uterus complete their differentiation program. Our results demonstrate the ability of chemokines present in the uterine compartment, such as CXCL12/SDF-1, CX3CL1/Fractalkine and CXCL10/IP10, to support peripheral blood NK cell migration through endothelial and stromal decidual cells as well as the ability of freshly isolated decidual NK cells to migrate through stromal cells. Notably, we observed that this capability is significantly increased when progesterone cultured stromal cells were used as substrate and strongly correlated with the ability of progesterone to positively modulate the expression of chemokines on stromal cells. Furthermore, we found that when peripheral blood human NK cells were allowed to take an intimate contact with decidual stromal cells or with autologous adherent decidual cells, they acquired a chemokine receptor pattern that mostly resembles that of decidual NK cells. Collectively these results strongly suggest that during early pregnancy, peripheral blood NK cell

recruitment to the uterus contributes to the enhancement of NK cell numbers in this non-lymphoid organ where NK cells undergo reprogramming of their chemokine receptor pattern and acquire a specific phenotypic and functional profile to ensure a good outcome of pregnancy. This work was supported by grants from the European NoE EMBIC within FP6 (contract number LSHN-CT-2004-512040).

MS7-3

NK cell regulation and Type I propensity in women with recurrent pregnancy losses and infertility of multiple implantation failures

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Women with recurrent pregnancy losses and infertility of multiple implantation failures demonstrate propensity to type I immune response. Peripheral blood Th1/Th2 cell ratios were significantly elevated in these women as compared to those of normal controls. Recently, we have studied type I immune response in NK cell populations, and T regulatory (Treg) cell populations. Treg cells were reported to have a major role in auto-reactive T and B cell regulation, and maintenance of a pregnancy. Treg cells were studied in women with reproductive failures using multiple markers; Foxp3+ cells were subdivided according to CD4 and CD25 expression, i.e., CD4+Foxp3+, CD4-Foxp3+, CD25+Foxp3+, CD25-Foxp3+, CD4+CD25+Foxp3+, and CD4+CD25-Foxp3+ cells. The main population of Foxp3+ cells was CD4+Foxp3 cells in both controls and the patients. The proportion of CD4+Foxp3+ Treg cells in CD4+ cells is significantly decreased in women with RSA and multiple implantation failures as compared to controls ($p < 0.05$ each). The ratio of CD4+CD25+Foxp3+/CD4+ Treg cells is significantly correlated with that of CD4+CD25+/CD4+ cells. This finding supports the notion that T cell dys-regulation is already present in early stage of T cell differentiation in women with recurrent pregnancy losses and multiple implantation failures. Propensity to type I immune response was also present in NK cell

population. The level of CD56^{bright}/IFN- γ ⁺/TNF- α ⁺ cells (%) was significantly higher in women with RSA ($P < 0.05$) and implantation failures ($P < 0.01$) as compared with that of normal controls. The level of CD56^{bright}/IL-4⁺/IL-10⁺ cells (%) was very low ($< 2\%$) in all groups, but was significantly lower in women with RSA than that of normal controls ($P < 0.05$). The TNF- α /GM-CSF expressing CD56^{bright} cell ratio was significantly higher in women with RSA ($P < 0.05$) and implantation failures ($P < 0.01$) as compared with that of normal controls. We concluded that NK1 shift in peripheral blood NK cells was identified in non-pregnant women with RSA and implantation failures. Propensity to type I immune response is present in T and NK cell population of women with recurrent pregnancy losses and multiple implantation failures. It is noteworthy that T cell dys-regulation exists in early stage of T cell differentiation.

MS7-4

Contributions of endometrial lymphocytes to angiogenesis during pregnancy

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Problem: Commercial meat pigs experience two waves of spontaneous fetal loss during early and mid gestation of their 114-day pregnancy. Poor blood supply of the placenta is considered a participating cause. We hypothesize that endometrial angiogenesis at implantation sites is driven by products from recruited lymphocytes that promote gestational success and differs between sites with fetuses that are healthy or undergoing an early arrest.

Method of Study: Gilts (virgin or gestation days 20, 30, 50 ($n=4$ /group)) were studied. Expression of Vascular endothelial growth factor (VEGF), VEGF-receptors (I, II), Placenta growth factor (PlGF), Hypoxia inducible factor (HIF-1 α), interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) was quantified by real time PCR in 500-laser capture microdissected endometrial lymphocytes (LY) or endothelial cells (EC) and compared with intact endometrium or trophoblasts. VEGF family related proteins were evaluated

by immunohistochemistry and special stains were used to examine endometrial vasculature.

Results: LY, EC, endometrial biopsies and trophoblasts expressed all VEGF members and HIF-1 α but relative transcription varied with gestational age and implantation site viability. LY, EC and endometrium obtained from arresting attachment sites had fewer transcripts than samples from healthy sites. Endometrial angiogenesis appeared to be more robustly promoted by LY than by trophoblasts in healthy sites. Elevated transcription of IFN- γ and TNF- α accompanied fetal arrest. In arresting sites EC were irregularly shaped and arteries were narrower, surrounded by elastin fragment and heavy collagen deposits.

Conclusion: Peri-implantation and midgestation fetal losses can be lymphocyte-promoted processes involving endometrial vascular failure and coordinated onset of inflammation.

MS7-5

Significance of soluble HLA-G detection in follicular fluids and embryo supernatants in IVF/ICSI cycles

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Problem: To document if soluble (s)HLA-G detection in individual follicular fluids (FF) or embryo culture supernatants (ES) could be a marker of potential of clinical pregnancy.

Methods: 117 Individual follicular fluids, 309 Day-2 embryo culture supernatants (Day-2ES)(Center 1) and 276 day-3 embryos supernatants (Day-3 ES) (Center 2) were collected, and blindly analyzed. Traceability from fully documented embryos was established up to the implantation for 117 FF, 122 Day-2 and 103 Day-3 ES using the Web-based database (Medifirst). sHLA-G was blindly detected using an optimized chemiluminescent Elisa Assay (Center 3).

Results: sHLA-G in FF, D2 and D3 ES were detected in respectively 96%, 23% and 68% of the samples. FF and Day2- sHLA-G concentrations were not correlated. In the FF, the sHLA-G concentrations do not significantly influence the implantation rates. sHLA-G detection significantly increases from D-2 to D-3 ES tested. IR were higher in detectable groups compared to undetectable groups in Day-2 ES (39 versus 20%, p=0.01) and Day-3 ES (15 versus 5%, p=0.08).

Conclusion: sHLA-G in the FF does not influence the implantation in contrast to the amount detected in ES. The low percentage of detection on Day-2, even faithful for implantation, is a limit for a clinical application. From Day-3 embryo supernatant, sHLA-G detection could however be very useful marker for implantation prognosis but power of the study should be increased. Our study is therefore still ongoing. This work was supported by the European network of excellence EMBIC (contract 512040).

MS7-6

A model for uterine NK cells as killers not builders in pregnancy

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Problem: Organ-specific innate immunity at the maternal-fetal interface is thought to be crucial for fetal development and immunoprotection, but its potential contribution to the pathophysiology of pregnancy-associated disorders has received little study. Despite a volume of work accumulated on uterine NK (uNK) cells, their precise role as a friend or a foe to pregnancy remains a mystery, in particular when associated with the absence of a pregnancy compatible anti-inflammatory milieu. In this regard, our recent work provides an experimental evidence for detrimental effects of cytotoxic uNK cells resulting in fetal resorption in IL-10^{-/-} mice. This raises important questions about the functional spectrum of uNK cells. It is possible that these cells

participate in late pregnancy complications such as premature delivery, pre-eclampsia, intrauterine growth restriction (IUGR) and respond to inflammation and infections at the time of hormonal anomalies. Premature birth in response to inflammation provides an experimental avenue to address these possibilities. Preterm birth remains an enigmatic reproductive disorder with a poorly understood etiology, except for a strong association with intrauterine infections.

Methods: Timed pregnancies were established involving wild type and IL-10^{-/-} mice. LPS (0.5 µg/mouse) was administered i.p. on gd 14 and animals were either euthanized on gd 16 or allowed to deliver. Utero-placental tissue was either fixed for immunohistochemistry or processed for mononuclear cell isolation and immune profiling. Pregnancy outcome was recorded at the time of the delivery.

Results: We show that uNK cells mediate inflammation-induced preterm delivery in the absence of IL-10. Administration of low doses of lipopolysaccharide (LPS) late in gestation results in preterm delivery in IL-10^{-/-}, but not wild type, mice. Although uNK cells are nearly absent during later stages of pregnancy, LPS-induced preterm birth in IL-10^{-/-} mice is associated with an increased number and placental-infiltration of cytotoxic uNK cells, and extensive placental cell death. Depletion of NK cells or TNF-α neutralization in LPS-treated IL-10^{-/-} mice restores term delivery. Furthermore, *in vivo* inactivation of TNF-α prevents uNK cell infiltration and placental cell apoptosis.

Conclusions: These results imply that a regulatory axis involving uNK cells, TNF-α, and IL-10 plays a crucial role in the pathophysiology of inflammation-induced preterm birth.

MAIN SESSION 8: REGULATORY MECHANISMS IN PREGNANCY

MS8-1

Trophoblast-immune interactions: the role of TLRs during pregnancy

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Immune cells such as macrophages and neutrophils are present at the implantation site as early as the first week of implantation. This evidence suggests that the innate immune system is not indifferent to the fetus and may play a role, not only in host protection to infections, but also in the fetal-maternal immune adjustment. A significant aspect in this process is the establishment of an adequate microenvironment that will promote cell growth and inhibit harmful inflammatory immune reactions. Although there is a large body of evidence supporting the semi-allograft theory, which focuses mainly on studying the process of tolerance towards paternal antigens presented by the blastocyst, we have shown that the invading trophoblast actively recruits maternal immune cells following the trophoblast's recognition of bacterial or viral components. Trophoblast cells express the receptors to recognize pathogen-associated molecular patterns (PAMPs) expressed by bacteria, virus, parasites, and fungi. These transmembrane pattern-recognition receptors (PRRs), namely Toll-like receptors (TLR), are key players in the activation of the innate immune system. All ten human TLRs are expressed by the placenta, and stimulation of TLR-4 by LPS or stimulation of TLR-3 by Poly(I:C) (a synthetic analogue of viral sdRNA) leads to an increased production of pro-inflammatory chemokines by human trophoblast cells. We hypothesized that during implantation an inflammatory environment is necessary for the attachment and invasion of the blastocyst¹⁶. Therefore we propose the existence of an 'inflammatory-mediated embryo implantation' condition which is dependent on the proper "education" of the innate immune system by the trophoblast. In turn, the recruited monocytes respond to the trophoblast in a supportive manner by producing and secreting factors, such as cytokines and chemokines, which promote trophoblast survival and function.

MS8-2

Role of male seminal factors in establishing maternal immune tolerance for pregnancy

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Problem: The conceptus expresses paternal antigens including MHC but is protected from immunological attack by an active state of maternal immune tolerance. The sequence of events during early pregnancy required to activate tolerance is not clear. We hypothesise a central role for male seminal fluid in provision of male antigens and immune-deviating cytokines in priming the female immune response for pregnancy.

Methods: T-cell transgenic mouse models with ovalbumin (OVA) as a model paternal antigen were utilized to investigate the occurrence, kinetics and MHC restriction of paternal antigen activation of the female immune system after exposure to seminal fluid. GM-CSF null mutant mice were used to evaluate the role of the dendritic cell cytokine GM-CSF in regulating the female response.

Results: OVA-reactive OT-I and OT-II T-cells administered to female mice mated with males constitutively expressing ovalbumin in seminal fluid express activation markers and proliferate in lymph nodes draining the uterus prior to embryo implantation. Activated T-cells are restricted to maternal H-2 haplotype. The amplitude of the T-cell proliferative response is diminished when female mice are GM-CSF deficient.

Conclusions: Our data show that paternal antigens delivered within semen stimulate female T-cell activation and proliferation, via maternal dendritic cells using both MHC class I and class II restricted pathways. GM-CSF is an essential cytokine for efficient uterine dendritic cell antigen processing and presentation. This provides a mechanism whereby semen exposure constitutes the initial priming event for paternal antigen recognition and tolerance induction in early pregnancy, and uterine GM-CSF synthesis induced by seminal fluid signalling amplifies this response.

MS8-3

How do the CD4+CD25+Foxp3+ regulatory T cells maintain tolerance?

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Problems: How do organ-specific CD4+ CD25+ Foxp3+ regulatory T cells (Treg) influence autoimmune disease induction and progression?

Method of Study: From normal B6AF1 mice, Treg from lymph nodes (LN) draining ovary, prostate or lacrimal gland were quantified for suppression of oophoritis, prostatitis and dacryoadentitis in d3tx mice. On d3tx B6AF1 mice, and d3tx mice expressing transgenic spermatid-specific ovalbumin (OVA) plus transgenic TCR to pOVA (DO11.10), the total and pOVA specific Foxp3+ Treg cells in LN and target organs were characterized, and their *in vivo* function determined.

Results: Treg in regional LN of normal mice were 15 to 50 times more efficient in suppressing autoimmune disease of the draining organ than the Treg from non-draining LN. D3tx led to severe autoimmune disease of ovary and testis. Unexpectedly, their total and antigen-specific Treg were increased rather than decreased in both LN and target organ. They were functional; thus Treg depletion accelerated disease in d3tx mice. In transgenic model, severe orchitis coincided with emergence of IL17-producing antigen-specific effector T cells.

Conclusions: Antigen specific Treg in normal mice are strategically positioned to anticipate and avoid potential concomitant autoimmunity in organ specific infections or other danger signals. In autoimmune d3tx mice, Treg are increased and not reduced despite disease progression, thus the disease is due to Tregs' failure to contain effector T cells.

MS8-4

Indoleamine 2,3-dioxygenase in immunoregulation

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The tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) is reported to be critically implicated in the mechanism of maternal immunotolerance of the semiallogeneic fetus. Immunoregulation via IDO is based on inhibition of T and NK cell proliferation by tryptophan catabolism exerted by macrophages and dendritic cells. IDO is induced by interferons, engagement of CD200R and CTLA-4. Hormones such as hCG, oestrogen and progesterone contribute to the regulation of IDO. We discuss findings pertaining to the role of IDO in pregnancy, its location at the feto-maternal interface, systemic induction of IDO in pregnancy and the association of IDO to spontaneous abortion and preeclampsia. Whereas there is a large body of evidence supporting the relevance of IDO as a key immunoregulatory factor in feto-maternal tolerance, open questions remain concerning as to its role.

MS8-5

Regulatory benefits of pregnancy

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Rheumatoid arthritis frequently improves during pregnancy, which is followed by an exacerbation of the disease postpartum. Whilst the underlying mechanism remains elusive, it is clear that female sex hormones play an important role. We have made two observations that might shed light on the modulation of the autoimmune response. Firstly, that the regulatory T cell population expands during pregnancy to prevent an immunological rejection of the fetus and secondly that regulatory T cell function is altered in rheumatoid arthritis patients. Here we examine whether the pregnancy-induced temporary remission in human disease also occurs the mouse model. We were able to demonstrate that pregnancy protects mice from the onset of collagen-induced arthritis. Next, we used adoptive transfer experi-

ments to assess the potency of regulatory T cells from different sources in preventing arthritis. We found that regulatory T cells from mice in pregnancy-induced remission from arthritis are more potent in preventing the onset of disease than those from control mice. This suggests that pregnancy not

only increases the number of regulatory T cells per se, but specifically induces antigen specific tolerance, which at least in part is due to regulatory T cells.

MAIN SESSION 9: INFLAMMATION AND IMPLANTATION

MS9-1

Chemokine tuning at the fetal – maternal interface

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Fetal loss in animals and humans is frequently associated with inflammatory conditions. D6 is a promiscuous chemokine receptor with decoy function, expressed in lymphatic endothelium, that recognizes and targets to degradation most inflammatory CC chemokines. Here, we report that D6 is expressed in placenta on invading extravillous trophoblasts and on the apical side of syncytiotrophoblast cells, at the very interface between maternal blood and fetus. Exposure of D6(-/-) pregnant mice to LPS or anti-phospholipid autoantibodies results in higher levels of inflammatory CC chemokines and increased leukocyte infiltrate in placenta, causing an increased rate of fetal loss, which is prevented by blocking inflammatory chemokines. Thus, the promiscuous decoy receptor for inflammatory CC chemokines D6 plays a nonredundant role in the protection against fetal loss caused by systemic inflammation and anti-phospholipid antibodies.

are not selective for foreign targets and may attack also host cells. We sought to determine whether this situation is encountered at the placental level where C can be activated by structural changes that occur in the decidua including vascular remodelling and trophoblast invasion.

Method of study: Specimens of first trimester placenta were analysed for complement deposition by immunohistochemical analysis. Trophoblast and decidual endothelial cells (DECs) were isolated and examined for synthesis of C components by RT-PCR and ELISA and for the ability of these components to promote trophoblast-DECs interaction.

Results: Most C components are synthesized by decidual macrophages, endothelial and stromal cells, and trophoblast. DECs, unlike the endothelial cells of non pregnant uterus, synthesize C1q, which is secreted and deposited on the cells surface. The bound C1q was found to promote adhesion of DECs to trophoblast that express a C1qR for the collagen portion of the molecule. Another interesting finding was that DEC express also C7 on their surface which functions as a down-regulator of DEC activation. C components were documented on the decidual vessels of spontaneous abortions.

Conclusions: C exhibits novel functions at fetomaternal interface.

MS9-2

The complement system at the fetomaternal interface: friend or foe?

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Problem: The Complement (C) system is an essential component of innate immunity and acts as a double-edged sword providing an effective defence against harmful agents and representing a real danger for tissues under conditions of unrestricted activation. The targets are identified by recognition molecules and neutralized by biologically active products released following C activation through one of the three pathways. Unfortunately, these products

MS9-3

Before Antigen induces Immunity: V-ATpase and inflammation during pregnancy

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Pregnancy is an example of normal, physiological generation of immune tolerance. Recent data suggests that the embryo itself may induce the immune response in order to produce an inflammatory response and finally, vascularization and growth. In normal development, the implanting embryo actively responds to its surroundings by inducing inflammation, and this causes the maternal tissue to

release cytokines. This occurs prior to and without antigenic differences between mother and embryo. Our studies focus on a specific component of a vacuolar ATPase molecule (V-ATPase) called (a2-VAT-Pase) previously identified as Regeneration and Tolerance Factor. V-ATPases are critical to implantation and successful pregnancy. a2-VATPase, as the a2 isoform of the "a" subunit of V-ATPase is critical for a functional V-ATPase. In immune cells and actively growing cells, such as fetal and maternal cells found at the site of blastocyst implantation, a2-VAT-Pase is present both within the cell and on the cell surface. a2-VATPase has two potential mechanisms by which it may regulate local inflammatory processes and apoptotic processes, both of which are vital to implantation. First, when present in the cell membrane, a2-VATPase regulates ATP hydrolysis at the cell surface, which in turn regulates the P2X7 channel. Upon activation, P2X7 triggers the release of IL-1 β and the onset of apoptotic events. Secondly, soluble a2-VATPase (the N-terminal region) has been shown to induce IL-1 β production directly in monocytes and T cells. During pregnancy, a2-VAT-Pase regulates IL-1 β , apoptosis and the immune response in fetal and maternal tissues prior to antigenic stimulation.

MS9-4

Anti-phospholipid antibody-mediated fetal loss: direct effect of the autoantibodies on trophoblast and decidual cells

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aPL are risk factor for recurrent pregnancy loss/complications. The mechanisms of aPL-mediated pregnancy failure are still matter of research. Although aPL are associated with thrombosis, thrombotic events cannot explain all the miscarriages. There is evidence for a direct in vitro aPL effect on the trophoblast as shown by their binding, reduced proliferation, hCG release, in vitro invasiveness, adhesion molecule expression and increased apoptosis. Such a direct reactivity is supported by the expression of beta₂GPI on human trophoblast. aPL/anti-

beta₂GPI abs also bind to human decidual/endometrial cells in vitro. The binding specificity for beta₂GPI is supported by the reactivity of affinity purified polyclonal APS IgG and of human monoclonal IgG anti-beta₂GPI Ab (IS3). Once bound the Abs induce an increase in ICAM-1 expression. Altogether these findings suggest a complex direct action of aPL/anti-beta₂GPI Abs on trophoblast/decidual cells with functional pathogenic effects. Preliminary evidence shows that it is possible to interfere with these mechanisms. TIFI is a 20 a.a. peptide able to inhibit the binding of beta₂GPI to EC and to prevent thrombosis induced by anti-beta₂GPI Abs in mice; incubation of trophoblast/decidual cells with serial concentrations of TIFI inhibited the anti-beta₂GPI Ab binding. Lower inhibitory effects were found by incubating the cells with TLR-4 ligand antagonist, or anti-annexin A2 antibody. Interestingly, upregulation of ICAM-1 on decidual cells was also inhibited by these molecules. As a whole these findings suggest that the binding of anti- β_2 GPI Abs to decidual cells involves β_2 GPI, annexin A2 and TLR-4.

MS9-5

Inflammation induces pregnancy failure and growth restriction: complement inhibition as a possible target for therapy

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Appropriate complement inhibition is an absolute requirement for normal pregnancy. Uncontrolled complement activation in the maternal-fetal interface leads to fetal death. Here we show that complement activation is a crucial and early mediator of pregnancy loss in two different mouse models of pregnancy loss. Using a mouse model of fetal loss and growth restriction (IUGR) induced by antiphospholipid antibodies (aPL), we examined the role of complement activation in fetal loss and IUGR. We found that C5a-C5aR interaction and neutrophils are key mediators of fetal injury. Treatment with heparin, the standard therapy for pregnant patients with aPL, prevents complement activation and protects mice from pregnancy complications induced by aPL, and anticoagulants that do not inhibit complement

do not protect pregnancies. We identified release of TNF- α and tissue factor (TF) expression as critical intermediates that act downstream of C5 activation. In an antibody-independent mouse model of spontaneous miscarriage and IUGR (CBAXDBA) we also identified C5a as an essential mediator. Complement activation caused dysregulation of the angiogenic factors required for normal placental development. In CBAXDBA mice, we observed inflammatory infiltrates in placentas, functional deficiency of free vascular endothelial growth factor (VEGF), elevated levels of soluble VEGF receptor 1

(sVEGFR-1, also known as sFlt-1; a potent anti-angiogenic molecule), and defective placental development. Inhibition of complement activation blocked the increase in sVEGFR-1 and rescued pregnancies. Our studies in antibody-dependent and antibody-independent models of pregnancy complications identified complement activation as the key mediator of damage and will allow development of new interventions to prevent pregnancy loss and IUGR.

MAIN SESSION 10: IMMUNOLOGY OF PREECLAMPSIA

MS10-1

Evolution of ideas on physiopathology of preeclampsia

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Hypertensive disorders of pregnancy (HDP) remain a major public health problem as they concern approximately 10% of human pregnancies. From 1900 to the 1980's, numerous studies confirmed what has been well-known for 3 centuries for eclampsia : HDP were « the disease of primigravidae » with a protective effect of any kind of preceding pregnancy (including miscarriages or abortions). At the end of the 1970's and during the 1980-90's, 3 major advances occurred in comprehension of preeclampsia : First, unique to human pregnancy, there are two trophoblastic invasions, one at time of implantation (like all mammals), and a second very deep one at the end of the first trimester of pregnancy ; it is the defect of this second wave which implies a rise of maternal blood pressure to try to bring supplies to the foetus through the shallow implantation. Second, in severe preeclampsia and eclampsia, all maternal symptoms are due to a global endothelial cell disease (glomeruloendotheliosis, liver and cerebral vasculitis); since then, researches are focused on a possible « factor X » cause of this vasculitis. And third, epidemiological studies showed that the father may play a role : HDP can be a disease of first pregnancy at a level of a couple, and not only a « disease of primigravidae » (concept of « primipaternity »), with a protective effect of a long sexual cohabitation before conception in new couples (sperm exposure ?). The last decade has seen an explosion of immunological studies on preeclampsia with notable discoveries : the immunological role of the sperm, the crucial involvement of Natural-Killer cells in implantation, notably for the indispensable angiogenesis surrounding the trophoblast, the possible mismatching among couples concerning paternal HLA-C and maternal receptors. These aspects will be discussed with possible perspectives on further researches.

MS10-2

NK cells and the maternal inflammatory response in pre-eclampsia

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The immunological interaction between the mother and fetus has classically been thought of as one between paternal antigen and the maternal T cells. However, the MHC antigen expression on human trophoblast and the immune cell populations present in the decidua suggest that this interaction primarily involves decidual NK cells rather than T cells, and this is supported by new functional studies. It is also becoming apparent that the maternal systemic immune response in pregnancy (Th1/Th2 shift) primarily involves NK cells. Aberrant NK cell activation both locally in the decidua and systemically in the maternal blood may be the cause of pre-eclampsia.

MS10-3

Circulating microparticles and immunoregulation in pregnancy and pre-eclampsia

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Cellular particles are ubiquitously shed from cell membranes or secreted as endocytic vesicles (exosomes). Microparticles are larger and are generated during apoptosis or necrosis. Exosomes are smaller and released from late endosomes after cell stimulation. Both membrane particles and exosomes can be detected in the circulations of non-pregnant and pregnant women. In non-pregnant individuals microparticles are increased in conditions associated with systemic inflammation such as sepsis or metabolic syndrome. During pregnancy they are also associated with pre-eclampsia. They include particles

derived from platelets, endothelium and various leukocytes. In pregnancy syncytiotrophoblast-derived microparticles are derived from the syncytial layer of the placenta. Syncytiotrophoblast membrane microparticles (often called STBMs) interact with both immune and endothelial cells. They may contribute to the systemic inflammatory response of both normal and pre-eclamptic pregnancies, although inhibitory activity has also been described. Moreover, the repeatedly observed downregulation of T cell activity in pregnancy may in part be caused by trophoblast derived exosomes. A suggested mechanism is deletion of activated T cells, which express Fas ligand by Fas expressing exosomes derived from trophoblast.

MS10-4

Combinations of maternal KIR genes and fetal HLA-C alleles influence reproductive success: studies of pre-eclampsia, fetal growth restriction and recurrent miscarriage

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Problem: Maternal uterine NK cells have KIR receptors whose ligands include HLA-C present on fetal trophoblast cells. We have shown that in pre-eclampsia (PE), where defective placentation occurs, there is an association with certain combinations of maternal KIR and fetal HLA-C genotypes (Hiby *et al.* J Exp Med 2004;200: 957–65). Inadequate placentation is also the primary pathogenesis in many pregnancies where fetal growth is severely compromised (FGR) or results in recurrent miscarriage (RSA). We have now investigated whether KIR/HLA-C associations are important in all three conditions.

Method of Study: We genotyped two independent cohorts of pre-eclamptics, a group of mothers with

severely growth restricted babies (FGR) but no PE, women with unexplained recurrent miscarriage and controls for KIR and HLA-C. HLA-C was typed as group 1 and 2, the allotypes distinguished by NK cells through their KIR receptors.

Results: In all three types of reproductive failure an association with specific KIR/HLA-C combinations was found. Specifically, a fetal HLA-C2 group combined with the lack of the activating KIR for HLA-C2, KIR2DS1, was a significant risk factor. Birth-weight *per se* was influenced by this combination and there was a gene dosage effect for the HLA-C2 group.

Conclusions: A specific combination of maternal and fetal genotypes, which would result in strong NK cell inhibition, contributes to reproductive failure. The genes involved are expressed on NK cells (KIR) with ligands on trophoblast (HLA-C) and suggest that placentation and reproductive performance is regulated by the innate immune system.

MS10-5

Different cytokine genotypes characterizes preterm labour with and without premature rupture of membranes

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Problem: Spontaneous preterm labour with premature rupture of membranes (PROM) or without (SPT) is mediated by an inflammatory reaction involving the cytokine network. Considering the fact that some cytokine gene polymorphism may affect the level of cytokine production, the purpose of this study was to investigate the relationship between the polymorphisms of the genes coding for TNF- α (-308 G \rightarrow A), IL-10 (-1082 G \rightarrow A), IL-6 (-174 G \rightarrow C), and IFN- γ (+874 A \rightarrow T) and SPT and/or PROM.

Method of Study: Genotyping was performed in 233 mulatto women with history of spontaneous preterm labour, being 126 without (SPT) and 107 with premature membrane rupture (PROM); and in 104 ethnically matched women without obstetric pathology. Data were analyzed by the χ^2 or Fisher

exact test, with the level of significance set at $p \leq 0.05$.

Results: We observed a significant association between high producing T/T IFN γ genotype and PROM, but no differences between SPT and controls genotypes. In addition, our data showed increased frequency of high producing TGF- β genotypes and a trend towards association with the high production IL-10 genotypes in SPT women as compared to PROM group. There was no association between SPT

or PROM and TNF- α (-308 G \rightarrow A) and IL-6 (-174 G \rightarrow C).

Conclusions: The observed associations are relevant not only as risk factors but also because they represent another piece of evidence that relevant differences underlines the development of SPT and PROM. Supported by FAPEAL

MAIN SESSION 11: CLINICAL IMMUNOLOGY

MS11-1

New Insights into the Etiology and Diagnosis of Implantation Failure

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Problem: Implantation is essential for survival of all mammals. Failure of implantation is the most common cause of lack of successful pregnancy after *in vitro* fertilization and embryo transfer. Successful implantation requires invasion of the blastocyst into the endometrium where it must stimulate its own blood supply. In order for it to invade, the trophoblastic cells proliferate, induce a paracrine apoptotic reaction and secrete proteases capable of digesting the endometrial extracellular matrix (MMPs). Activation of MMPs at the implantation site is stimulated by the serine protease, plasmin, the production of which is regulated by plasminogen activator inhibitors (PAI). The p53 tumor suppressor gene is a potent inducer of apoptosis, regulates cell growth and induces angiogenesis. Vascular Endothelial Growth Factor (VEGF) is the best characterized regulator of angiogenesis.

Method of Study: Buccal swabs were obtained from 70 women with history of recurrent implantation failure after IVF/ET and 73 control women. DNA was extracted from the buccal swabs and analyzed for the presence of the PAI 4G/4G, VEGF -1154A/A and Arg/Pro genotypes.

Results: The frequency of homozygosity of the PAI 4G/4G and VEGF -1154A and Pro 72 genes were significantly higher among women experiencing recurrent pregnancy loss compared with control women.

Conclusion: Implantation is a complex process with redundant mechanisms such that a defect in one mechanism will not result in implantation failure. The challenge is to identify the combination of defects that will lead to implantation failure.

MS11-2

Autoantibodies to factor XII and kininogens in patients with recurrent pregnancy losses

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Problem: Plasma contact proteins such as factor XII and high molecular weight kininogen have anticoagulant and profibrinolytic activities *in vivo*. Recently, numerous studies have suggested the associations between contact protein deficiencies and recurrent pregnancy losses (RPL), and between autoantibodies to contact proteins and RPL. We reported that certain antiphosphatidylethanolamine antibodies (aPE) are not specific for phosphatidylethanolamine (PE) *per se*, but are directed to PE-binding plasma proteins, kininogens (Sugi and McIntyre. *Blood* 1995; 86: 3083–89). We also reported a statistically stronger association between RPL and kininogen-dependent aPE than between RPL and antibodies to anionic phospholipids for early gestational losses. Later, it has been reported that antibodies to factor XII showed a strong and statistically significant association with RPL.

Method of study: We used synthetic peptides in inhibition and direct binding studies to identify the antigenic binding sites of autoantibodies to factor XII and kininogens in RPL patients.

Results: Among plasmas from 24 RPL patients who were positive for kininogen-dependent IgG-aPE, 17 (70.8%) recognized Leu331-Met357 which is located on the carboxyl-terminal portion of kininogen domain 3 and known as the major kininogen cell attachment site. Among plasmas from 17 RPL patients who were positive for autoantibodies to factor XII, 13 patients (76.5%) recognized the amino-terminal heavy chain region (amino acids 1–30) of factor XII, which is known as binding site to platelet GP Ib-IX-V complex.

Conclusions: Both autoantibodies to factor XII and kininogen-dependent aPE may block factor XII- and kininogen-binding to GP Ib-IX-V complex and augment thrombin-induced platelet aggregation.

MS11-3

Praneem polyherbal vaginal tablets cure 70–95% of vaginosis /vaginitis caused by a large spectrum of Reproductive tract and sexually transmitted infections

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We reported the development of a polyherbal formulation (Praneem) with action on a wide spectrum of genital pathogens (Talwar, et al. *Am. J. Reprod Immunology*, 2000;43, 144–151). It inhibited the growth of N.gonorrhoea including antibiotic resistant strains, MDR E.coli, Staph. aureus; Candida albicans, C.tropicalis and C.krusei. Studies at Johns Hopkins showed prevention of infection by vaginal route of Herpes simplex –2 and chlamydia trachomatis in progestin primed mice by Praneem and studies at Institut Pasteur Paris & Conrad Lab Norfolk indicated its

virucidal action on HIV. After preclinical toxicology, Phase I clinical trials documented the safety of Praneem (Bagga et al *Trans. Royal. Soc. Trop. Med. Hygiene* 2006;100:1164–1167; Joshi et al *Trans. Roy. Soc. Trop. Med. Hygiene*, 2005; 99: 769–774.) Phase II efficacy studies at Postgraduate Institute of Medical Education & Research followed by multicentric trials in 6 centres of the Indian Council of Medical Research have shown the ability of Praneem to cure 70% of patients suffering from abnormal vaginal discharge after 7 night use of Praneem intra-vaginally. The cure rate was above 90% on extension of treatment to 14 days. 100% of women with *Trichomonas vaginalis*, 70% with microorganism causing vaginosis & 82% with *Candida* infections were rendered free of infection, after 7 days of treatment. Praneem is currently in Phase III large scale trials and may become available for public use in the near future.

ABSTRACTS OF SELECTED ORAL PRESENTATIONS AND POSTERS

ICRI-07-1-1

Complement receptor gC1qR expression and distribution in bovine spermR Valeris¹, P Villamediana¹, A Quintero², O Parra³, J Sandoval³, B Ghebrehwet⁴¹Animal Biotechnology Research Unit. Faculty of Veterinary Sciences and Experimental Faculty of Sciences. University of Zulia. Maracaibo. Venezuela;²Animal Production Research Unit (UNIPA). Faculty of Veterinary Sciences. University of Zulia. Maracaibo. Venezuela;³Clinical Research Unit. Faculty of Veterinary Sciences. University of Zulia. Maracaibo. Venezuela;⁴Department of Medicine. State University of New York at Stony Brook. Stony Brook. NY, U.S.A

Problem: Determining the identity of the molecules involved in sperm-oocyte interaction could lead to breakthroughs that will eventually allow the design of more efficient *in vitro* fertilization protocols in domestic species, especially in bovines. In addition, this could also serve as a basis for predicting sires' reproductive performance. Recently, complement receptor gC1qR/p33, a multi-compartmental and multifunctional protein that recognizes the globular heads of C1q, has been shown to play a role in reproduction in humans. The objective of this research was to determine the gC1qR distribution and expression on plasma membrane in bovine sperm before and after heparin treatment.

Method of Study: capacitation of sperm derived from frozen-thawed bovine semen was induced through heparin incubation and its effectiveness was assessed with a CTC stain. Subsequently, indirect immunofluorescence assays were conducted with mAb (60.11, anti-gC1qR) to assess gC1qR distribution and expression. Data were statistically analyzed through χ^2 test and Fisher's exact test.

Results: Data analysis demonstrated that gC1qR is expressed on the plasma membrane of bovine sperm. gC1qR showed a capacitation-related redistribution, migrating to the acrosome region: 175 sperm in heparin-incubated aliquots vs. 109 in control ($P < 0.05$, $n = 300$) showed fluorescence over the acrosome region. This distribution is similar to that reported in humans. Similarly, either gC1qR expression or its accessibility to antibodies increased after capacitation. This also correlates with what has been observed in humans.

Conclusion: These results suggest that gC1qR could participate in primary sperm-oocyte interaction in bovines.

ICRI-07-1-2

The NKT cells in the follicular fluid and peripheral blood of infertile women: a pilot studyJ Krizan¹, L Cuchalova², J Madar², J Rezacová², P Sima¹, M Kralickova³¹Department of Immunology and Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, v.v.i., Videnska 1083, Prague 4, the Czech Republic;²Institute for Care of Mother and Child, Department of Immunobiology, Podolske nabrezi 157, Prague, the Czech Republic;³Department of Obstetrics and Gynecology, University Hospital, Faculty of Medicine, Charles University, Capkovo namesti 1, Pilsen, the Czech Republic

Problem: The NKT cells represent important branch of cell immunity. There are several papers documenting the changes of NKT cells in peripheral blood and decidua, which may suggest that, may participate in regulation of the Th1/Th2 balance and the cytokine production at the fetomaternal interface during implantation.

Method of study: The aim of this study was to identify the differences in abundance ratios (relative proportions) of NKT cells in follicular fluid and peripheral blood in groups of infertile women, in which the infertility was caused by the ovarian disorder, the tubal closure, and the endometriosis. The group of healthy women, in which the cause of infertility relied on their partners, formed the controls. The flow cytometry has been used.

Results: This is the first report proving the presence of NKT cells in follicular fluid. No significant changes in relative proportions of NKT cells in follicular fluid were detected in all infertile groups including controls. Increased relative proportions of NKT cells in peripheral blood were documented only in women with infertility caused by tubal closure.

Conclusions: These findings suggest that NKT cells outside of follicle fluid (from peripheral blood) could be connected with tubal closure infertility without affection of relative proportions of NKT in follicular fluid.

ICRI-07-1-3

Live birth from preantral follicle oocytes in cryopreserved mouse ovaries after IVG, IVM and IVF

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Problem: As ovaries are large stores of oocytes, cryopreservation is a feasible method for preserving female reproductive potential, especially in young patients with cancer or in women at risk of premature ovarian failure. In this study we examined whether cryopreserved oocytes from mice could develop into live pups after in vitro growth (IVG) and in vitro maturation (IVM).

Method of study: Cryopreservation was carried out by a vitrification method. For IVG, oocyte-granulosa cell complexes (OGCs) from mouse ovarian preantral follicles were cultured for 6 days. The grown OGCs were transferred to a maturation medium to assess nuclear maturation and then subjected to in vitro fertilization (IVF).

Results: Histological examination revealed that the cryopreserved ovaries could preserve good morphology compared with fresh ovaries. Among OGCs recovered from cryopreserved ovaries, 44.7 % achieved to maturation after IVG/IVM. After insemination 57.3 % of the oocytes were fertilized. Subsequently, 50.7 % of the fertilized eggs developed into a blastocyst stage. These rates are not statistically different when compared to those obtained from fresh ovaries. Moreover, two live pups were obtained following transplantation.

Conclusions: This study demonstrated that OGCs in preantral follicles recovered from cryopreserved ovaries maintained competence to grow and mature in vitro, and the mature oocytes successfully developed into offspring following IVF-ET. These results encouraged us to apply the ovarian cryopreservation and in vitro culture to preservation of fertility.

ICRI-07-1-4

Distribution of beta-tubulin in pathological human sperms

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Problem: The cytoskeletal proteins in the sperm are in connection with fertilization and motility. We compared the distribution and amount of tubulin in spermatozoa from men with normal and pathological spermograms.

Method of study: Human sperm were divided into five groups. Immunocytochemical and biochemical methods were used in our experiments. The acrosome state of sperm was tested by MoAb (Hs-14) against intraacrosomal proteins. For detection of cytoskeletal proteins the following MoAbs were used: TU-01 (IgG1) against the N-terminal structural domain of α -tubulin, TU-12 (IgM) against the C-terminal structural domain of β -tubulin and TU-06 (IgM) against the N-terminal domain of β -tubulin, TUB 2.1 (IgG1) against the C-terminal structural domain of (-tubulin and antibody 18D6 (IgG1) against the N-terminal peptide of (-tubulin. The concentration of proteins was evaluated by modification of the Smith's method (BCA).

Results: Striking differences in immunofluorescence (IF) staining were observed with anti- β -tubulin antibody TU-12 that decorated the sperm tail and the equatorial segment of the head. The epitope was located in the (-tubulin region (426–435). Quantitative immunoblotting revealed differences in the amount of tubulin among men with normozoospermia and those with pathology. The evaluation of IF results with TU-12 antibody and control anti-acrosomal antibody showed statistically significant differences in the staining of sperm head among the samples of normal and pathological sperm.

Conclusions: The results suggest that tubulins, which are involved in functional organization of the sperm, could be useful biomarkers of the pathological sperm state and might be applied in human medicine and assisted reproduction.

ICRI-07-1-5

Biochemical nature of boar sperm antigen with functional activity in primary sperm-zona pellucida binding

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Problem: Investigations on specific and functionally active sperm antigens would bring about the elucidation of the mechanisms of gamete interaction and help the search to new approaches for prognosis and regulation of fertility. Previously, we have produced a monoclonal anti-boar spermatozoa antibody 4B12 that might affect the primary sperm-zona pellucida binding. It was shown that the 4B12 antigen was shared between different animal species and secreted both in the testis and epididymis.

Results: Using indirect immunofluorescence (IIF) and ELISA tests, here we provide evidence that Mab 4B12 reacts with ejaculated and capacitated boar spermatozoa, the reaction being intensified after the capacitation. By Sephadex G-200 chromatography of NP40 extracts from capacitated spermatozoa the Mab 4B12 cognate antigen is found to be eluted in the ascending part of 4th protein fraction. The results from SDS-PAGE and Western blot of the fraction containing 4B12 antigen show specific reactivity of the antibody with two protein bands of 85 kDa and 330 kDa. The results from Western blot of NP40 extracts from capacitated spermatozoa show reactivity of the antibody with the same proteins and two additional protein bands of 165 kDa and 300 kDa. In Western blot of extracts from epididymis Mab 4B12 reacted with 85 kDa protein.

Conclusion: It is suggested that Mab 4B12 identified an aggregated polypeptide molecule originating from a precursor protein (Mr 85 kDa) present on epididymal spermatozoa, that is exposed on sperm acrosome after ejaculation and intensified concomitantly with the occurrence of capacitation. Future investigations are needed to explain the mechanisms of involvement of 4B12 functional domains in gamete interaction.

ICRI-07-1-6

Increased expression of secretory actin-binding protein (SABP) on human spermatozoa is associated with poor semen quality

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Problem: Antibodies to human sperm are useful diagnostic reagents for detection of changes in sperm protein expression and their relationship with sperm defects and male infertility. The specificity of Hs-16 monoclonal antibody and the localization and frequency of the occurrence of Hs-16-recognized protein on human spermatozoa were investigated.

Method of study: The specificity of Hs-16 antibody was analyzed by the Western blotting technique and MALDI mass spectrometry. In indirect immunofluorescence Hs-16 antibody was used to test sperm ejaculates.

Results: The Hs-16 antibody detected a human sperm and seminal plasma protein, which was determined as secretory actin-binding protein (SABP). This specificity was also verified by colocalization of SABP and actin on spermatozoa with Hs-16 and anti-actin antibodies, and partial colocalization of these proteins was found. SABP was localized on the sperm tail, mainly in the midpiece of the tail. Other parts of spermatozoa were labeled with lower frequency. A significant difference was found in SABP expression between sperm from men with normal spermograms and men with pathological spermograms. Increased expression of this protein was observed in men with asthenozoospermia.

Conclusions: Hs-16 antibody reacts specifically with SABP. SABP can serve as a marker of defective sperm and may be associated with fertility failure.

ICRI-07-1-7

Sperm associated antigen 9 (SPAG9) – potential candidate molecule for fertility control: Immunogenicity study of recombinant SPAG9 in a bonnet macaque (*Macaca radiata*)

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Problem: The human sperm-associated antigen 9 (hSPAG9) is of special interest attributing to the findings indicating that SPAG9 is an acrosomal molecule. SPAG9 is not only restricted to acrosomal compartment but also persists in equatorial segment post-acrosome reaction, which is a key location in sperm-egg interaction.

Method of study: Cloning and expression of hSPAG9 was done in prokaryotic expression vector pET 28b(+). The recombinant construct (pET28b-hSPAG9) was expressed in *Escherichia coli* and purified using Ni-NTA resin. Immunization were carried out with recombinant hSPAG9 (rhSPAG9) adsorbed on alum in macaques. Gel electrophoresis, immunoblotting, indirect immunofluorescence assay, and hamster sperm egg penetration assay were carried out to characterize the anti-SPAG9 antibodies.

Results: Immunogenicity studies in macaques were carried out with recombinant SPAG9 adsorbed on alum, which resulted in high titers of anti-SPAG9 antibodies as determined by enzyme-linked immunosorbent assay (ELISA). Immunoblotting analysis employing anti-rhSPAG9 antibodies generated in monkeys indicated that antibodies specifically reacted with native SPAG9 from macaque and human sperm and rhSPAG9 protein. Furthermore, indirect immunofluorescence experiments demonstrated SPAG9 localization in the acrosomal compartment of macaque and human sperm. In addition, monkey antibodies against rhSPAG9 significantly inhibited the human spermatozoa adherence or penetration in zona-free hamster oocytes.

Conclusion: These results demonstrate that rhSPAG9 adsorbed on alum is highly immunogenic in

subhuman primate model and therefore represents a suitable sperm-based vaccine immunogen for fertility trials in macaque.

Acknowledgement: This work was supported by grants from Department of Biotechnology, Government of India and Mellon foundation and CONRAD, USA.

ICRI-07-1-8

Expression of costimulatory molecules, chemokine receptors and proinflammatory cytokines in dendritic cell population in normal and chronically inflamed rat testis

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Problem: Dendritic cells (DC) are potent antigen presenting cells and presentation of self antigens by DC is likely to play an important role in the initiation of autoimmunity and its progression. Our recent characterization of testicular autoantigens in experimental autoimmune orchitis (EAO), a model of chronic testicular inflammation, prompted us to investigate the activation state of DC in normal and EAO rat testis. The activation state is crucial in determining the outcome of antigenic challenge viz the development of either T cell immunity or tolerance.

Method of study: DC were quantified immunohistochemically in normal, control and EAO Wistar rat testis using the DC specific antibodies Ox-62 and CD11c followed by stereological analysis. The expression of CD80, CD86 and MHC class II on DC was analyzed by flow cytometry. Moreover, testicular DC from normal and diseased rats were isolated (97%) by magnetic bead separation followed by FACS and expression of chemokine receptors (CCR2 and CCR7), IL-10 and IL-12 was determined by RT-PCR.

Results: The number of DC in EAO testes (ca. 7×10^5 /testis) increased significantly compared to

control rats (ca. 1×10^5 /testis). No significant differences in the expression of CD80, CD86 and MHC class II between groups was shown, however significantly elevated expression of CCR7 and a concomitant decrease of IL-12p40 subunit mRNA in the 50 day EAO group were evident. The CCR2 level in EAO animals was significantly decreased compared to adjuvant controls and expression for IL-10 and IL-12p35 subunit were detectable only in 50 day EAO group.

Conclusions: These data suggest that the DC in EAO testis have already processed (auto)antigens and are in a status to migrate to the local lymph nodes for T cell activation. They are in a ready migratory state, but functionally immature.

ICRI-07-1-9

ZP3-specific antibody is a vital component of virally vectored immunocontraception induced by a recombinant murine cytomegalovirus expressing zona pellucida 3 in mice

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Problem: A recombinant murine cytomegalovirus expressing zona pellucida 3 (rMCMV-ZP3) induces complete and irreversible sterility in immunocompetent BALB/c mice, markedly depleting ovarian follicles within 3 weeks of infection. It is likely that ZP3 antibody plays an important role.

Method of study: Immunoglobulin-deficient and control immunocompetent BALB/c mice were inoculated with a standard dose of MCMV-mZP3 vaccine. Male mice were introduced immediately and breeding was allowed to proceed for 100 days. The number of pups born during this time was counted and used to determine vaccine success. Alternatively, naïve females received a single intravenous inoculation of serum containing anti-ZP3 antibodies or a control serum, and males were introduced one day later. The time to first litter was recorded.

Results: Immunoglobulin-deficient females showed no reduction in fertility after inoculation with the vaccine and the ovaries appeared normal at the end of the experiment. Conversely, control immunocom-

petent mice were rendered completely sterile with almost complete follicular loss at 100 days post inoculation. The reduction in follicles at 21 days was accompanied by altered expression of surrogate markers of ovarian health: growth differentiation factor-9, connexin-43 and the homeobox gene, *nobox*. Additionally, immunoglobulin-deficient mice inoculated with anti-ZP3 antibodies showed a significant delay in the time before delivery of the first litter compared to controls. We are continuing to explore the effect of ZP3 antibody on the ovarian structure.

Conclusions: Antibody is vital for the success of rMCMV-ZP3-induced immunocontraception.

ICRI-07-1-10

Biological and Cytostatic effects of some animal and plant nucleases and their PEG-conjugates

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Problem: The antitumor and immunosuppressive effects of Bovine pancreatic ribonuclease (RNase A), Bovine seminal ribonuclease (BS-RNase), wheat leaf neutral ribonuclease (WLN-RNase) and Mung bean nuclease (PhA) both *in vitro* and *in vivo* were studied.

Method of study: RNase A and PhA enzymes were obtained from commercial sources whereas BS RNase and WLN-RNase were isolated in our laboratories from bull seminal vesicles and wheat leaves. Antiproliferative activity was tested on ML-2 cell line and immunosuppressive effect on human lymphocytes in mixed lymphocyte culture. The antitumor activity *in vivo* was tested in athymic nude mice bearing human melanoma tumor.

Results: The free RNase A and PhA-nuclease exerted very little antiproliferative and immunosuppressive activities in comparison to the effect of BS-RNase. Immunosuppressive effect of WLN-nuclease reached the same level as that of BS-RNase. However, after their conjugation to PEG these preparations evoked cytotoxic activity after i.t. or i.p. administration. The tumor size after treatment with PEG-RNase A

decreased by 70 %. The both free or PEG-conjugated BS-RNases decreased the tumor size also by the same way. The strongest aspermatogenic, embryotoxic and immunosuppressive effects, and antigenicity were proved by BS-RNase enzyme. The smallest side effects were demonstrated by PhA nuclease.

Conclusion: This paper shows for the first time that polymer conjugates of some plant RNases might be used as antitumor agents in treatment of human malignancies. Work supported by the Grant Agency of the Czech Republic no. 523/04/0755 and 521/06/1149. The authors are also grateful for the supporting to I RP I APG no. AVOZ 50450515 and partly by the Grant no. RA 8033-3 (Ministry of Health, Czech Republic) and by the Grant League against Cancer Prague.

ICRI-07-1-11

Soluble HLA-G as a marker of embryo quality in IVF cycles

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Problem: Identification of embryo quality and implantation potential markers remains actual for embryo selection in IVF cycles. The aim of this study was to evaluate the sHLA-G concentrations in day 3 embryos culture media droplets in relation to IVF cycles results.

Method of study: A total of 225 embryo culture supernatants from 50 patients were assayed. 120 embryos were selected for transfer in 50 IVF cycle. Double monoclonal sandwich immunoassay kit for quantitative measurement of s-HLA-G (Ex-Bio; Czech Republic) was utilized.

Results: In 19 (38,0%) treatment cycles pregnancy was achieved. Soluble HLA-G was detected in 49 (22,2%) of 225 samples. In 19 pregnant cycles 37 embryos were transferred, 13 (68,4%) were sHLA-G positive, the coincidence of high morphological score and sHLA-G was observed in 10 (27,0%) of them. At least one sHLA-G positive embryo was detected in 12 (63,1%) cycles. In 31 non-pregnant cycles 83 embryos were transferred, 13 (15,6%) were sHLA-G positive, the coincidence of high morphological score

and sHLA-G were found in 7 (8,4%) of them. At least one sHLA-G positive embryo was detected in 7 (22,5%) cycles ($p < 0,05$ for all data). Pregnancy rate in the cycles, where sHLA-G positive embryos were selected for transfer was 52,6% (10 cases). In the cycles without sHLA-G positive embryos pregnancy rate was 29,0 % (9 cases). In the cycles, were high morphological score and sHLA-G secretion coincides, the pregnancy rate was 63,3%.

Conclusions: Evaluation of sHLA-G status to morphological criteria may be useful as an additional clinical tool for embryo selection.

ICRI-07-1-12

Effect of progesterone on the expression of HLA-E in human choriocarcinoma cell line

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Problem: Among non-classical class I HLA molecules, HLA-E plays an important role in the recognition of extravillous trophoblast cells by maternal cells as a major ligand of CD94/NKG2 receptor on NK cells. But it is still unknown how its expression is regulated. Progesterone is considered to be an essential hormone for the maintenance of pregnancy as an immunomodulator at fetomaternal interaction.

Method of Study: We investigated whether progesterone has a regulatory effect on HLA-E expression in JEG3, human choriocarcinoma cell line. HLA-E mRNA amount in cultured JEG-3 cells was assessed by real-time PCR and surface expression was analyzed by flowcytometry with HLA-E specific monoclonal antibody, 3D12.

Results: HLA-E mRNA was remarkably highly expressed one hour after the addition of progesterone and that response was diminished by RU486, progesterone antagonist. The increase of HLA-E surface expression was also observed from two to four hours after the addition of progesterone.

Conclusion: Progesterone was suggested to upregulate HLA-E expression. This finding provides a new insight into immunomodulatory function of progesterone at fetomaternal interaction.

ICRI-07-1-13

Murine cytomegalovirus clear MHC class I molecules from the cell surface of infected cell and retrieve them in the juxtannuclear pre-late endosomal compartment

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Problem: Murine cytomegalovirus (MCMV) down-regulate MHC class I molecules (MHC-I) from the cell surface at early stages of infection. The aim of our study was to compare intracellular localization of MHC-I on uninfected and MCMV-infected mouse embryonal fibroblasts (MEF).

Methods of study: MEFs were infected with deletion mutant of MCMV devoid of viral Fc receptor gene, and intracellular localization and trafficking of MHC-I molecules was determined by confocal microscopy. Cell surface expression of MHC-I molecules was followed by flow-cytometry and intracellular stability by immunoprecipitation after cell surface biotinylation. MHC-I were labeled with monoclonal antibody MA-215 (K^d) and 34-5-8s (D^d). In order to define a compartment wherein MHC-I are accumulated colocalization with a Golgi marker (GM130), an early endosome marker (EEA-1), a lysosomal marker (LAMP-1) and with antibody against TfR (R-17) were used. Microtubular network was disrupted by nocodazole (6.6 μM).

Results: We have found that K^d and D^d molecules, which are present at the surface of uninfected cells, are down-regulated from the surface of infected cells. These molecules can be found accumulated in juxtannuclear region of infected cells in large vesiculo-tubular structures. K^d and D^d molecules colocalized with GM130, R-17 and in small amount with LAMP-1 and EEA-1. In order to dissect whether these molecules are retrieved by the Golgi or in endosomal vesicles, we used nocodazole to disperse juxtannuclear organelle, including the Golgi complex. After nocodazole treatment, Golgi complex was dispersed into vesicles containing K^d and D^d molecules, however almost half of retained molecules did not colocalize with GM130.

Conclusions: From these results we can conclude that virus down-regulates the MHC-I from the cell

surface of infected cells into the juxtannuclear vesicles that are after sorting endosomes in the endocytic pathway (EEA-1) and before late endosomes (Lamp1). These vesicles are outside of the recycling route (TfR-). Significant amount of the nascent MHC-I molecules are, in addition, retained in the Golgi complex. Supported by Croatian Ministry of Science, Grant No 0620238-0223.

ICRI-07-1-14

Expression and release of the NKG2D ligands RAET1/ULBPs from human placenta

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Problem: Placenta plays a crucial role in the adaptation of the mother to the fetus and can be viewed as an immunomodulatory organ. We have previously shown that the NKG2D ligands MICA and MICB are expressed and released by the syncytiotrophoblast (STB) of human placenta. In this study we analysed the second family of NKG2D ligands, the RAET1/ULBP family, in human normal placenta.

Method of study: Early and term placenta samples were analysed by quantitative RT-PCR and immunohistochemistry (IHC) for mRNA and protein expression of RAET1/ULBP molecules. Exosomal fraction was isolated from placental explant cultures by ultracentrifugation and analysed by dot blot, western blot and immunoflow cytometry with latex microbeads for exosomes and RAET1/ULBP molecule expression.

Results: mRNA for all five members of the RAET1/ULBP family is expressed by early and term placenta. IHC studies showed ULBP2 expression by STB. RAET1/ULBP molecules were present in the supernatant of placental explant cultures and in the exosomal fraction that was extracted from the culture supernatants.

Conclusions: The syncytiotrophoblast of human placenta from normal pregnancy expresses the RAET1/ULBP family of NKG2D ligands. Analyses of supernatants from placental explant cultures indicate that these molecules are released in soluble form.

Expression and release of RAET1/ULBP might have a two fold purpose – RAET1/ULBP expression might target stressed/infected or damaged STB for elimination by NK cells while released RAET1/ULBP molecules might engage and downregulate the NKG2D receptor in a similar way as sMIC molecules do. Further studies are in progress to elucidate this proposal.

ICRI-07-1-15

Interactions between HLA-G molecule and decidual natural killer cells of human first trimester pregnancy

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Problem: Non-classical MHC-I molecule HLA-G, selectively expressed on invading extravillous cytotrophoblast, is in close contact with decidual CD56⁺ NK cells infiltrating first trimester decidua. We showed recently that exposure to HLA-G positive trophoblast altered their phenotype and function (decidual NK cells were able to induce FasL protein expression in close contact with HLA-G molecule and it represented dominant mechanism of cytotoxicity for K562-HLA-G cells). Therefore, our aim was to investigate further influence of this molecule on decidual NK cells.

Methods of study: Decidual tissue was minced, treated with collagenase and passed through density gradient. After centrifugation, decidual mononuclear cells were collected. Intracellular expression of cytolytic mediators, NK cell activating receptors and Th1 cytokines was determined in decidual CD56⁺ cells after their 18 hour-culture with K-562-PWD cells and K-562-HLA-G transfectants at 37°C (1:1). Immunohistology for FasL and TRAIL was performed.

Results: After overnight culture, decidual NK cells decreased their content of perforin in close contact with HLA-G molecule, comparing to their co-culture with K-562-PWD. We also observed HLA-G mediated decrease of activating NK receptor expression (NKG2D, NKG2C, NKp44, NKp46) as well as Th1 cytokine expression (IFN- γ , IL-18, TNF- α) in

decidual NK cells. FasL and TRAIL were detected on paraffin-embedded first trimester decidual tissue sections as well as FasL by using Alexa 555.

Conclusions: HLA-G decreased expression of cytolytic mediators, NK cell activating receptors and Th1 cytokines in decidual CD56⁺ cells. Since expression of HLA-G molecule is limited to gestation, it seems to be important in the regulation of various decidual NK cell functions. The experiments are partially financed by Croatian Ministry of Science (Grants No. 0620402-0376 and 0379) and EMBIC project, European FP6, NoE, LSHM-CT-2004-512040.

ICRI-07-1-16

L-carnitine induces establishment or aggravation of endometriosis in a murine experimental model

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Problem: Accumulated evidence indicates an association between endometriosis and alteration of the humoral and cell-mediated immunity. While the role of L-carnitine in the regulation of energy metabolism is well established, it was recently recognized to modify the immune response in mice and induce infertility following a phenotypic pathology similar to clinical endometriosis. This study was designed to test whether L-carnitine may aggravate an already established endometriotic state.

Method of Study: Transient endometriosis was established upon intra-peritoneal administration of human endometrial cells represented by the cell lines Ishikawa and MFE-319 to female mice with or without L-carnitine. Serum and peritoneal fluid (PF) were collected 5, 10 or 60 days later and submitted to ELISA and immunofluorescence experiments for the detection of cytokines and immune cell populations. The levels of free or acyl-L-carnitine were measured in the serum and PF by specific radioassays.

Results: Human endometrial cells were shown to implant to the mouse uterus providing a hemorrhagic phenotype of the uterus and alter the cellular and cytokine profile of serum and PF, resembling the phenotype of endometriosis. Administration of L-carnitine to these experimental models further increased the accumulation of endometriosis-specific

cell populations and cytokines in the PF and serum, while decreasing the acyl-L-carnitine showing that the exogenously administered L-carnitine had been metabolically processed.

Conclusions: These results indicate that L-carnitine not only facilitates the establishment of endometriosis in the female organism but can also aggravate an already existing endometriotic state, recommending careful prescription of this agent during the reproductive period of life.

ICRI-07-1-17

Fully conformed MHC class I molecules and empty L^d molecules follow different routes in the endocytic pathway

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Problem: MHC class I molecules are present in the cell as "full" trimolecular complexes composed of properly folded heavy chain, β_2 -microglobulin (β_2m) and peptide (conformed MHC-I molecules), or "empty" heavy chains devoid of peptide and often associated with β_2m (no-conformed MHC-I molecules). The aim of our study was to compare the endocytic trafficking of conformed and non-conformed murine MHC-I molecules (K^d, D^d and L^d) in non-polarized cell lines.

Methods of study: Endocytic trafficking of MHC-I molecules was followed in the model of spontaneous internalization after surface labeling with monoclonal antibodies (mAbs) or biotin. Intracellular pathway was dissected by treatment with inhibitors of vesicular transport and characterized by confocal microscopy and colocalization with endosomal markers. Surface biotinylation and immunoprecipitation was used to determine the kinetics of MHC-I degradation.

Results: In the steady state, conformed and non-conformed MHC-I molecules partially colocalized. After internalization, both conformed and non-conformed molecules were found in sorting endosomes (SEs), but after that point they separated. Conformed MHC molecules were recycled through the juxtanuclear recycling endosomes, whereas non-conformed MHC-I molecules were directed to

an acidic endosomal retention compartment. From the retention compartment empty L^d molecules were directed either into lysosome for degradation or into the exocytic pathway.

Conclusions: Conformed and non-conformed MHC-I molecules travel together to the sorting endosomes and separate into the different routes after they leave SEs. Conformed MHC-I molecules travel the recycling route and gradually enter the degradation pathway. Non-conformed molecules are retained in a late acidic multivesicular compartment from which they are sorted into the exocytic route or degradation pathway. The experiments are partially financed by Croatian Ministry of Science Grant No. 0620238-0223 and EMBIC project, European FP6, NoE, No. 512040, LSHM-CT-2004-512040.

ICRI-07-1-18

Incompatibility of HLA-G may be associated with effectiveness of husband's lymphocyte therapy in recurrent spontaneous abortion

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Problem: Controversy in the effectiveness of husband's lymphocyte therapy (LT) for recurrent spontaneous abortion (RSA) may be caused by confusion in selection of cases. If HLA-G and -E expressed on trophoblasts is involved in RSA, compatibility/incompatibility of HLA-G alleles between couples with RSA may influence the clinical outcome of LT in RSA. In the present study, allelic distribution of HLA-G and -E antigens was investigated in view of clinical outcome of LT to obtain information about the selection of cases effective for LT.

Method of study: A total of 30 couples with RSA in which wives received LT were genotyped for HLA-G and HLA-E under the permission of Ethical Review Committee of Tokai University. Among the RSA couples, 20 cases were successful to obtain live-birth, while early abortion was observed in 10 cases. All the aborted fetuses were subjected to karyotyping to

ensure no chromosomal abnormalities. Number of HLA-G or -E alleles shared by couples and number of incompatible paternal alleles were compared between the live-birth and abortive groups.

Results: The number of HLA-G alleles shared by couples was significantly greater in abortive group than in the live-birth group ($p=0.006$ by Fisher's test), while no difference was observed in the number of shared HLA-E alleles. The number of incompatible paternal HLA-G alleles was significantly greater in the live-birth group as compared with the abortive group ($p=0.010$ by Fisher's test), while no difference was noted for HLA-E alleles. These observations suggested that, first, sharing by more than one HLA-G alleles between couples with RSA attenuated effectiveness of LT, and secondly, compatibility of HLA-G in the RSA couples was associated with the failure of LT.

Conclusions: Incompatibility of HLA-G alleles from husband to wife should be considered in standardizing the indication of LT. A large cohort study with strict indications is needed to confirm the observations in this study.

ICRI-07-1-19

Immune regulation in human IVF-pregnancy with semi-allogenic and allogenic fetuses

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Problem: In pregnancies achieved from oocyte donation fetal genome is allogenic in contrast to semi-allogenic in natural pregnancy. In spite of increased incidence of gestational complications relatively positive obstetric outcome of such IVF pregnancies put a question of additional regulatory mechanisms that support pregnancies with allogenic fetuses.

Method of study: 73 women: 23 - donation of oocytes (DO), 22 - IVF, 28 - natural pregnancy (NP) were studied in first trimester of pregnancy. Immunophenotyping of lymphocytes and intracellular production of cytokines were studied by multi-color flowcytometry.

Results: We found more high expression of chemokine receptors CXCR3 and CCR4 on CD4 T cells in DO pregnant women to compare with NP women, but only CCR4 expression on CD4 was higher in DO than in IVF women. Intracellular production of TNF- α , IFN- γ , IL-4 by CD4 T lymphocytes was higher in DO women than in IVF women. In IVF women intracellular production of IL-4 was lower than in DO women and NP women and there were no differences in NP and DO groups. In IVF women expression of activation marker CD69 on CD4+ T cells was lower than in DO women and NP women. In DO and IVF women levels of CD3+CD8+HLA DR+ were higher than in NP women.

Conclusions: Hyperactivation of Th1 and Th2 by allogenic fetus is specific for pregnancy achieved from oocyte donation. Preferable activation of Th2 in cases of oocyte donation reflected additional regulatory counteractive mechanisms that support pregnancy rate and positive obstetric outcome in spite of increased frequency of complications.

ICRI-07-1-20

Internalization and intracellular localization of HLA molecules

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Problem: MHC class I molecules could be found inside the cell and at the cell surface in the form of fully conformed trimolecular complexes (full molecules) and various conformers devoid of the antigenic peptides (empty molecules). In this study we compared endocytic trafficking and intracellular localization of fully conformed and non-conformed different HLA molecules (HLA-G1, HLA-E, HLA-B7 and HLA-Cw6).

Method of study: In our experiments we used murine fibroblastic J-26 cell line transfected with distinct HLA molecules (HLA-G1, HLA-E, HLA-Cw6 and HLA-B7) and human epithelial carcinoma (HeLa) cell line. Constitutive (after cycloheximide treatment) and induced (after mAb binding) internalization was followed by flow cytometry. Conversion of conformation was achieved by short low-pH treatment (45 sec; pH 2.0). Markers of intracellular compartments were used in order to distinguish intracellular localization

of HLA-molecules by confocal microscopy. The kinetics of degradation was determined by cell surface biotinylation and immunoprecipitation.

Results: We have found that all HLA molecules spontaneously internalized from the cell surface, and fastest kinetics was observed for HLA-Cw6 and HLA-E molecules. Typically, empty and misfolded conformations internalized faster than fully conformed HLA molecules. Triton-X-100 treatment removed all conformed HLA molecules from the cell surface indicating their localization in non-organized membrane domains, whereas non-conformed HLA molecules were resistant to this treatment, indicating their localization in lipid rafts. Inside the cell, conformed HLA molecules accumulated in the juxtannuclear region, whereas non-conformed molecules were retained in large multivesicular pre-late-endosomal structures. All investigated HLA-I molecules partially colocalized with EEA-1, besides conformed HLA-G1. There was no colocalization with Rab 5A, Lamp-1, as well as with Rab 5B (with exception for HLA-Cw6 molecules). Non-conformed molecules have shown weaker colocalization with TfR than conformed.

Conclusions: From our results we can conclude that non-classical HLA molecules undergo to the slow process of spontaneous internalization and recycling, and a small part of them enter the degradation pathway during each cycle. Non-conformed molecules are internalized with faster kinetics and directed into the large acidic retention vesicles that are before late endosomes in the degradation route of the endocytic pathway. The experiments are partially financed by Croatian Ministry of Science Grant No. 0620238-0223 and EMBIC project, European FP6, NoE, No. 512040, LSHM-CT-2004-512040.

ICRI-07-1-21

Significance of HLA genotyping to assess indication of alloimmunotherapy in recurrent spontaneous abortion

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Problem: Effectiveness of husband's lymphocyte therapy (LT) for recurrent spontaneous abortion

(RSA) is controversial. Since HLA locus is involved in maternal immune regulation, polymorphisms in HLA genes including HLA-G known to be exclusively expressed on chorionic tissue were investigated in association with effectiveness of LT in RSA.

Methods of study: A total of 42 patients with RSA who had received ALT and 23 control patients with no history of abortion were subjected to allelic genotyping of HLA-G, -E -A, -B, -Cw, and -DRB1 under permission of Ethical Committee of Tokai University. All the parents underwent karyotyping and when either parent of the couples displayed abnormal karyotype(s), the couple was excluded from the study. All the abortuses were subjected to karyotyping to exclude de novo chromosomal abnormality.

Results: HLA-G*010401 was found in all LT-ineffective cases, 100%, of which 51.9% in effective cases, and 56.5% in controls were found, respectively. A*2402 and DRB1*1502, in strong linkage disequilibrium with G*010401 in Japanese also showed significant association with ALT-ineffectiveness. Stratification analysis suggested that G*010401 and DRB1*1502 might play synergistic roles in ineffectiveness of LT in RSA. Though the mechanisms are unknown at present, it is shown that in the first, husbands' LT appears effective in RSA with negative G*010401, and secondly, several HLA genes such as A*2402 and DRB1*1502 may be involved in effectiveness so as to attenuate efficacy of LT.

Conclusion: HLA genotyping including HLA-G may provide us with a predictive marker of the effectiveness.

ICRI-07-1-22

Female reproductive steroid hormones influence the apoptosis and cytokine secretion by monocyte-derived mature dendritic cells

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Problem: Dendritic cells (DC) are important component in the control and modulation of the immune

response. It is generally accepted that during pregnancy the immune response is biased to Th2 dominance and both reproductive hormones and DC are involved in this process. The aim of this study is to search for interrelation between these factors.

Method of study: Peripheral blood monocytes were isolated from healthy donors and cultured to obtain monocyte-derived DC following the well established procedure. In parallel experiments isolated DC were cultured in the absence or presence of estradiol (E2) or progesterone (Pg). Apoptosis of cultured DC was assayed using Annexin V-FITC and propidium iodide kit (Sigma CO). Secretion of IL-6, IL-10, IL-12 cytokines by the cultured DC was assayed using ELISA kits (R&D Systems).

Results: It was established that both E2 and Pg added from the beginning of the culture had no effect on the percentage of apoptotic immature DC while a higher percentage of apoptotic cells was recorded in the population of mature DC at day 11-12th of culture. No effect of these hormones was detected when secretion of cytokines from immature DC was assayed. Contrary to that E2 and Pg seemed to up-regulate the secretion of IL-10 by mature DC.

Conclusions: Female reproductive steroid hormones cause changes in the cytokine profile secreted by mature DC and thus biased the immune response to Th2 dominance. The increased secretion of IL-10 is possibly related to the increased apoptosis of mature DC by autocrine regulatory mechanism.

cells cytotoxicity and prevents fetal rejection. This report production and characterization describe the PIBF-specific monoclonal antibody (MAB 3A6) which is to be used for detection of PIBF in biological fluids.

Method of study: Mice were immunized with fusion GST-PIBF protein produced by Ecoli transformed with specific vector kindly donated by Dr. J.Szekeres-Bartho (Pecz University, Hungary). After fusion with P3U1 myeloma cells hybridomas were selected which produced MABs reacting with the immunogen in enzyme immunoassay (ELISA), ECL blots, flow cytometry and immunofluorescence.

Results: After screening a hybridoma secreting MAB 3A6 was selected which reacted in ELISA against the fusion protein GST-PIBF but did not react against GST alone. This result was confirmed after testing the MAB 3A6 in ECL blotting experiments against normal human serum, bovine serum albumin, lysates from human organs. Positive reactions were recorded against the GST-PIBF fusion protein as positive bands were detected at 74 kDa. In attempts to detect native PIBF in biological fluids and placenta lysates positive stained bands were seen at 48 kDa in placenta lysate, only.

Conclusion: The MAB 3A6 is reacting against PIBF and could be used for testing the presence of this protein in various biological fluids.

ICRI-07-1-23

Generation and characterization of new monoclonal antibody against progesterone induced blocking factor

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Problem: One of the most important sex hormone which shows high local level during the early stages of pregnancy is progesterone (Pg). Peripheral and placental CD8+ T cells from pregnant exposed to Pg produce progesterone induced blocking factor (PIBF), a 34-kDa protein that decreases natural killer

ICRI-07-1-24

Endometriosis and infertility: mechanisms of development

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Problem: Association between endometriosis and infertility remains poorly understood. Autoimmune nature of endometriosis is actively discussed during past decades. But it is not clear yet: are autoimmune reactions attributed to endometriosis or infertility development? To distinguish between mechanisms of endometriosis and sterility development the comparative analysis of system and local immune response in fertile and infertile women with endometriosis was performed.

Method of study: The peripheral and peritoneal lymphocytes and macrophages phenotype and

intracellular cytokine production was examined using flow cytometry. mRNA expression of MMP-2 and TIMP-2 in monocytes/macrophages was assessed by real-time RT-PCR method.

Results: It was found that some changes of immune response were common to all women with endometriosis independently to fertility or infertility and were characterized by the impairment of cell differentiation and cell-cell interaction both at systemic and local level with elevation of IFN γ synthesis in peritoneal fluid. Fertile women with endometriosis didn't exhibit other prominent alterations. Infertile women with endometriosis additionally had high level of B-1 cells and lymphocytes synthesizing IL-4 and IL-6. Maximal level of peripheral B-1 lymphocytes was associated with negative prognosis for fertility recovery after laparoscopic treatment of endometriosis. Increase of peritoneal macrophages activation with elevation of IL-1 β intracellular synthesis and MMP-2, TIMP-2 mRNA expression was seen only in infertile women with endometriosis.

Conclusions: Mechanisms of endometriosis and infertility development are different. Impairment of fertility in women with endometriosis is defined by activation of autoimmune reactions and peritoneal macrophages. (The work was supported by Russian President grant MD-9533.2006.7).

ICRI-07-1-25

Cellular and molecular mechanisms of enhanced invasiveness of endometrial cells and its role in the pathogenesis of endometriosis

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Problem: The aim of this study was to estimate the cellular and molecular mechanisms of enhanced invasiveness of endometrial cells and its role in the pathogenesis of endometriosis.

Method of study: Eutopic and ectopic endometrium of 25 women with endometriosis and of 20 healthy women was examined. Real time RT-PCR method was used for assessment of mRNA for MMP-2, TIMP-2 and TGF β expression. Surface phenotype of endometrial stromal cells and lymphocytes was estimated by flow cytometry.

Results: MMP-2 mRNA expression was significantly enhanced in endometrium of women with endometriosis but this rise was more prominent in ectopic than in eutopic endometrium. TIMP-2 mRNA expression was more pronounced in eutopic endometrium. The level of TGF β mRNA expression and its reception by stromal cells and MNC was significantly raised in endometriotic lesions. The amount of regulatory T cells was elevated in the eutopic and ectopic endometrium of women with endometriosis. In the ectopic lesion the increase of CD49e expression by stromal cells and simultaneous decrease in integrin expression by MNC was found. Cytokines produced by MNC of endometriotic lesions regulated in vitro the expression of TGF β mRNA and integrin expression on the surface of endometrial lymphocytes of healthy women.

Conclusions: It seems that enhanced invasiveness of endometrial cells during endometriosis is due to the impaired balance of MMP-2/TIMP-2, which can be regulated by cytokine microenvironment. TGF β might be the main regulatory molecule influencing the MMP-2/TIMP-2 ratio and Treg accumulation in endometriotic lesions. (The work was supported by Russian President grant MD-9533.2006.7).

ICRI-07-1-26

Anti zona pellucida antibodies impair mouse folliculogenesis and oocyte meiotic maturation *in vitro*

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Problem: The mammalian zona pellucida, which is composed of strong immunogenic glycoproteins, is important because it allows a bidirectional communication between the oocyte and granulosa cells. Antibodies against zona pellucida may affect follicular development and meiotic maturation of oocytes.

Method of study: Antisera were prepared in rabbits by immunization with mouse ZP2 and ZP3 synthetic peptides. Preantral follicles were obtained from 16 day old BDF1 mice and cultured for 8 days in α -MEM plus 10% NRS (control), anti ZP2, or anti ZP3 antiserum. Then, follicles were transferred to

maturation medium (hCG and EGF) to evaluate mucification and oocyte maturation, or fixed for electron microscopic observation.

Results: During *in vitro* growth, follicle diameters were lower in anti ZP2 and ZP3 groups compared with control ($p < 0.05$). Antral formation rates of anti ZP2 and ZP3 groups (27/40) were lower than control (37/40) ($p < 0.01$). After *in vitro* maturation, anti ZP2 (23/40) and ZP3 (18/40) groups had lower mucification rate than control (35/40) ($p < 0.005$), but metaphase II oocyte rate was only lower in anti ZP3 (1/40) group ($p < 0.001$). Zona pellucida thickness was lower in anti ZP2 and ZP3 groups compared with control ($p < 0.005$). Electron microscopic micrographs showed less granulosa cell processes and gap junctions in anti ZP2 and ZP3 groups compared with control.

Conclusions: Anti zona pellucida antibodies impaired the formation of gap junctions between oocyte and granulosa cells. As a result, folliculogenesis and subsequent meiotic maturation of oocytes were also altered. In humans, ovarian dysfunction and infertility might be caused by anti zona pellucida antibodies by the same pathogenic mechanism.

ICRI-07-1-27

Direct maternal-fetal immune interactions are affected by allergy and parity of the mother

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Problem: Fetal life is a critical period for the development of immune function and maternal factors may influence immune programming. The current study was carried out to determine if maternal responses to fetal alloantigens during pregnancy are altered by factors which affect maternal cellular immunity such as allergy and previous pregnancies.

Method of Study: Blood was collected from 120 healthy pregnant volunteers at 20wks, 30wks, 36wks gestation and 6wks post partum. Cord blood was also collected. Mixed lymphocyte reactions were used to measure cytokine responses towards fetal alloantigen. Women were grouped according to a) presence/absence of allergic disease and b) number of previous pregnancies.

Results: The ratio of maternal Th-2 IL-13 / Th-1 IFN γ response to fetal alloantigen was higher during pregnancy than postnatally (4.24 ± 2.50 , 3.14 ± 2.36 respectively; $P = 0.036$). Women who had allergic disease had a higher IL-13/IFN γ ($P = 0.001$) ratio during pregnancy (5.13 ± 2.85) compared to non allergic women (3.09 ± 1.76). Similarly women who had experienced multiple pregnancies tended to have a higher IL-13/IFN γ ratio compared to those in their first pregnancy.

Conclusions: Pregnant women produced a Th-2 skewed cytokine response to fetal alloantigens which was enhanced by allergic status and parity. These factors have the capacity to influence the cytokine milieu experienced by the fetus and could therefore play a role in the development of immune mediated diseases in early life.

ICRI-07-1-28

Antigen-presenting function of testicular macrophages in contact sensitivity *in vivo*

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Problem: The aim of our research was to test how the treatment of mice with low dose of cyclophosphamide (CY) affects the antigen – presenting capacity of testicular and peritoneal macrophages in the model of contact sensitivity reaction (CS).

Methods of study: Two experimental protocols were used. In one, mice received *i.v.* affinity purified anti-TGF β or anti-IL-10 mAb or mixture of both mAbs followed by *i.v.* injection of TNP-substituted peritoneal (TNP-Mf) or testicular macrophages (TNP-TMf). Control animals received Mf only. In the second protocol macrophage donors were treated one day before cell harvesting with 50mg/kg of CY. Then these cells, after TNP-substitution were injected into naïve recipients (TNP-MfCY or TNP-TMfCY). In both protocols 7 days after Mf transfer recipient animals were tested for contact sensitivity reaction using ear swelling test.

Results: Recipients treated with anti-TGF- β mAb and injected with TNP-Mf or TNP-TMf expressed positive CS reaction (1.9 ± 0.8 and 6.1 ± 0.7

respectively) in comparison to control mice non-treated with antibody (0.3 ± 0.3 and 1.5 ± 0.2 respectively). Control recipients of TNP-Mf were additionally treated with mixture of anti-TGF- β and anti-IL-10 mAbs or with anti-IL10 mAb only and expressed positive CS reaction (6.1 ± 0.3 and 5.5 ± 0.8 respectively) in comparison to control mice non-treated with antibody (0.3 ± 0.3). Similarly, recipients of TNP-MfCY or TNP-TMfCY expressed strong CS reaction (4.9 ± 0.6 and 4.2 ± 0.6 respectively). Our previous *in vitro* experiments showed that Mf and TMf of mice treated with CY produce significantly less TGF- β and IL-10 than Mf of non-treated animals.

Conclusion: Our results demonstrate that *in vivo* neutralization of TGF- β and IL-10 by CY-treatment of Mf donors or by anti-TGF- β and anti-IL-10 mAbs in cell recipients significantly enhances the antigen-presenting capacity of Mf and TMf, thus suggesting that both TGF- β and IL-10 play an important regulatory role in the afferent limb of the CS reaction.

ICRI-07-1-29

The effects of non-steroidal anti-inflammatory drugs on inhibition of cell proliferation and induction of cytotoxicity, cell cycle arrest, and apoptosis in a human choriocarcinoma cell line

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Problem: Besides their ability to suppress inflammatory processes in several diseases, anti-inflammatory drugs have a reputation for reducing cancer cell growth and the risk of cancer occurrence. Since the interaction of anti-inflammation drugs with human choriocarcinoma has not been studied, in this study, we employed the human choriocarcinoma BeWo cell line as a model of choriocarcinoma to test the effect of the drugs.

Method of study: NSAIDs used in this study were aspirin, indomethacin, mesalamine and naproxen. The effects of NSAIDs on cell proliferation and cytotoxicity were assessed by MTT and LDH assays. Cell cycle progression was investigated using flow cytometric analysis. The induction of cell death was

determined using DNA fragmentation detection assays.

Results: All drug treatment resulted in inhibition of cell proliferation in a dose-dependent fashion ($p < 0.05$) with IC_{50} values of 116.57, 17.58, 192.54 and 81.13 $\mu\text{g/ml}$ for aspirin, indomethacin, mesalamine and naproxen, respectively. These drugs did not affect cell adhesion in the first 3 h of exposure. In addition, the inhibition of cell proliferation was accompanied by increased cytotoxicity. Analysis of the progression of the cell cycle showed that these drugs triggered G0/G1 and S phase arrest. Furthermore, these anti-inflammatory drugs induced apoptotic cell death as visualized by DNA laddering and *in situ* cell death detection techniques.

Conclusions: Cytotoxicity of these drugs on human choriocarcinoma is mediated by both cell cycle arrest and induction of cell death and this could have important implications for the use of anti-inflammatory drugs for treating or preventing choriocarcinoma.

ICRI-07-1-30

Studies on the androgen effect for the expression of macrophage inhibitory factor(MIF) and translationally controlled tumor protein(TCTP) in mouse Sertoli cells

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Objective: The role of androgen during spermatogenesis is still unclear. Previously, we reported that androgen increased MIF and decreased TCTP in mouse TM4 Sertoli cell line using SELDI-TOF mass spectrometry. In this study, we analyzed the mRNA expression of MIF and TCTP *in vitro* and *in vivo*.

Methods: 1) We analyzed the expression of MIF and TCTP using real-time PCR in TM4 cell line after treatment with or without dihydrotestosterone (DHT) 2) Balb/c male mice were injected with flutamide (anti-androgen receptor) (2mg/body weight g/day) for 4 weeks. Mice injected with corn oil were used as control. After 4 week-injection, testes were removed and frozen sections were prepared. Sertoli cells were collected using Laser micro dissection. The

expression of TCTP in Sertoli cells were analyzed using real-time PCR.

Results: 1) The expression of MIF was significantly increased after treatment with DHT. On the other hand, the expression of TCTP was significantly decreased. 2) After treatment with flutamide, the expression of TCTP was significantly increased.

Conclusion: Androgen affected the expression of MIF and TCTP *in vitro* and *in vivo*. Androgen seems to be involved in spermatogenesis by affecting the expression of molecules in Sertoli cells.

ICRI-07-1-31

Study on the survival role of ANP-cGMP-P and NO in human periovulatory follicle

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Problem: In IVF cycles the incidences of follicular cells apoptosis is high and corpus luteum formation failed due to immuno-endocrine factors. Disturbance in the level and function of intraovarian survival factors are suggested. Our recent studies showed the presence of Atrial Natriuretic Peptide - cGMP - Progesteron (ANP-cGMP-P) system in periovulatory human follicle.

Methods: We investigate the survival role of ANP-cGMP- P system including NO, in *in vitro* cultured human Granulosa Luteinized Cells (GLCs) isolated after ovum pick-up from follicular fluids of IVF patients with addition of: hANP, 2Bu-cGMP, SNP (stimulator of cGMP synthesis), L-NAME (inhibitor of NOS), AG (inhibitor of P production), RU486 (inhibitor of PR). The concentrations of ANP, P, NO and the level of apoptosis were measured.

Results: We found the positive relationship among the levels of ANP, P in supernatant of cultured cells and cGMP (after stimulation), as well as their negative effect on Caspase-3 activity. Moreover, we documented that the inhibition of P synthesis by AG and PR by RU486 led to increased Caspase-3 activity in GLCs. Exogenous cGMP did not influence ANP production, while the cGMP (induced by SNP) augmented ANP production in GLCs. The production of P was stimulated both by cGMP and ANP. Concerning

NO generation we found that SNP increased NO concentration in supernatant of cultured GLCs but exogenous cGMP and ANP did not influence NO concentration. The Caspase-3 activity clearly showed that ANP, P and NO have had highest antiapoptotic effect, probably mediated by cGMP (SNP vs. L-NAME: 0.081 ± 0.0193 vs. 0.298 ± 0.043 ; $P = 0.0001$).

In conclusion: The survival role of ANP-cGMP-P system and NO in periovulatory human follicle was revealed by modulation of their production and effect in cultured human GLCs.

ICRI-07-1-32

Expression of inducible microsomal prostaglandin E synthase in local endometriosis lesions

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Problem: Recently, an inducible microsomal human prostaglandin E synthase (mPGES) was identified. This enzyme converts the cyclooxygenase (COX) product prostaglandin (PG) H₂ to PGE₂, an eicosanoid that has been linked to carcinogenesis. We have reported increased expression of COX-2 mRNA in local lesions of endometriosis. To further elucidate this mechanism, of endometriosis and its malignant transformation, we investigated the expression and localization of mPGES.

Method of study: Samples were obtained from 28 patients with informed consent. Samples formalin fixed and paraffin embedded were served for immunohistochemistry. Immunostaining was carried out with a rabbit mPGES-1 anti-human polyclonal antibody. In 12 cases, expression of mPGES mRNA was examined by RT-PCR using total RNA extracted from frozen samples.

Results: Immuno-histochemistry revealed increased mPGES immunoreactivity in endometriosis samples compared with eutopic endometria. Immunoreactivity of mPGES was observed in both epithelia and stromal or inflammatory cells. Increased expression of mPGES-1 mRNA was detected in most of endo-

metriosis samples. There was no correlation between the immunoreactivity of mPGES-1 and serum levels of CA125, but a reverse correlation between immunoreactivity of mPGES in epithelia and AFS score was noted. Interestingly, immunoreactivity of mPGES-1 was more remarkable in epithelia of endometrial cysts with border-line malignancies.

Conclusions: Our results suggested that expression of mPGES in addition to COX-2 contributes to possible increased PGE₂ production in endometriosis patients. mPGES-1 may have critical role in tumor development and malignant transformation in endometrial cyst.

ICRI-07-1-33

IL-23 ,IL-27 and TWEAK :potential regulators in a murine abortion system

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Problem Many implantation defects /early abortion seem linked with deregulation of the IL-12/IL-15/IL-18 tripod, TNF/NK network at the interface. High TNF in the pre/ preimplantation uterus is difficult to reconcile with abortifacient effects in pregnancy. Searching for regulators of IL-12/IL-18 family, and TNF antagonists in implantation, we investigated "new members" of IL-12 family, IL-23 and IL-27, and TWEAK (Tumor Necrosis factor-like WEAK inducer of apoptosis acting with TNF as Yin and Yang of (innate) immunity).

Method: IHC in murine placenta/ decidua and RT PCR in CBA x DBA/2 or BALB/c murine combinations days 0 -12,5

Results: IL-23 peaked at day 9.5 in NON ABORTING combination, a peak difficult to explain except by invoking feed back on EB13. Most important, modest, but real IL-27 hyper expression was seen in the CBA x DBA/2 mating as early as D4.5, becoming very marked by days 6.5- 9.5 TWEAK expression was deficient (6.5- 7.5) in DBA/ 2, mating, markedly expressed in BALB/C ones. A 1st peak of expression exists on days 4.5 5.5 in BOTH cases. resorption window.

Conclusion: Tweak is likely an important protection against TNF in implantation, explaining embryo survival in a TNF rich environment, with lack of pro-

tection in abortion prone mating. Data are compatible with a much earlier activation of inflammation / abortion initiation(day 4.5) than the classic "resorption window" early NK /mph activation described by Baines. Possible links with complement activation recently described by Girardi will be discussed. Neutralisation experiments and preliminary studies in human, will, time permitting be presented.

ICRI-07-1-34

Effect of leukocyte therapy on alteration of cytokine production and its relation to pregnancy outcome

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Problem: Recurrent spontaneous abortion (RSA) is defined as three or more sequential abortion below the twentieth week of gestation. Different cytokines have been recognized as important regulators of pregnancy events. Considering the important role of Th1/Th2 balance in pregnancy outcome a successful treatment for RSA should be able to make a significant change in Th1/Th2 balance and a shift away from Th1 responses. Although paternal leukocyte immunization has been used for treatment of RSA for two decades, there is controversy about mechanisms and effectiveness of this method. Thus the aim of the present study was to investigate the influence of paternal lymphocyte therapy on the Th1 shift and the effect of such changes on pregnancy outcome.

Method of Study: Twenty five Iranian non-pregnant women with RSA and sixteen non-pregnant control women with at least two successful pregnancies were included in this study. All cases were followed after leukocyte therapy for pregnancy outcome. Mononuclear cells from women were co-cultured with the husband's mononuclear cells before and after immunotherapy. The levels of TNF- α and IFN- γ (as Th1 or pro inflammatory cytokines) were checked on culture supernatant by ELISA method.

Results: The mean concentration of IFN- γ was significantly decreased after immunotherapy (P = 0.0001), but the mean concentration of TNF- α was significantly decreased only in women with successful outcome (P = 0.0001).

Conclusion: The results of this investigation confirm the role of TNF- α in RSA and also candidate the assessment of TNF- α production as a valuable prognostic parameter for the occurrence of abortion after leukocyte therapy.

ICRI-07-1-35

Trophoblast cells increase the proliferation and the IL-10 production of decidual T cells

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Problem: The cytokines produced by decidual T cells (IL-10, IL-4, LIF) could be important for the maintenance of pregnancy (Piccinni *et al.*, *Nat Med*, 1998; 4: 1020-1024). The factors present in the microenvironment of the decidual T cells, as molecules expressed and/or produced by trophoblast, could be responsible for the T cell cytokine profile.

Method of study: Insolubilized anti-CD3 antibody-activated CD4+ T cell clones generated from decidua of voluntary abortion were cocultured in the presence of irradiated trophoblast cells for 5 days. The proliferative response of the T cell clones in response to trophoblast cells has been evaluated and the levels of 27 cytokines in the supernatants of the anti-CD3-activated decidual T cell clones in response to trophoblast were measured by Luminex technology.

Results: We found that trophoblast cells increased significantly the T cell clones proliferation and that trophoblast cells were able to increase significantly the production of IL-10 by the decidual T cells. When the decidual T cell clones and the trophoblast cells were cultured with a permeable transwell support, that allow the passage of soluble molecules but avoid the contact between the T cells and trophoblast cells, the levels of IL-10 decreased significantly.

Conclusion: Thus, IL-10 produced by decidual T cells depends from the molecular contact of the trophoblast cells with the decidual T cells. The next step of our study would be to try to identify some of

the trophoblast membrane molecules responsible for the modulating activity of the trophoblast on the decidual T cells.

ICRI-07-1-36

Expression of surface CD1d in the extravillous trophoblast cells of early gestation placenta is downregulated in a manner dependent on trophoblast differentiation

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Problem: CD1d is a specific ligand for the invariant Valpha24Vbeta11-natural killer T (iNKT) cells that play an important role in placental development during early human pregnancy. The localization and regulation of placental CD1d expression remain unclear.

Methods: Immunohistochemistry of human placenta at first trimester of pregnancy was performed to assess the localization of CD1d at maternal-fetal interface. CD1d expression on trophoblast cells was quantified by flow cytometry analysis to confirm its decrease along with differentiation into extravillous phenotype. Quantitative RT-PCR of CD1d was performed to demonstrate the change of CD1d expression at transcription level. The trophoblast cells were exposed to TGF-beta1 or to IFN-gamma to examine their effect on CD1d expression.

Results: CD1d was shown to be present in villous and extravillous cytotrophoblast (EVT) but not in syncytiotrophoblast or decidual cells. CD1d immunoreactivity in EVT cells decreased with EVT differentiation. Flow cytometry of cultured trophoblast cells confirmed cell-surface expression of CD1d that decreased with time in culture. Quantitative-RT-PCR revealed that these changes in CD1d expression occur at the level of transcription. TGF-beta1 suppressed, while IFN-gamma induced, CD1d expression in a dose-dependent manner. Monoclonal antibody neutralization of TGF-beta1 inhibited its suppressive effects on CD1d expression.

Conclusions: The alteration in cell-surface expression of CD1d in trophoblast cells accompany

trophoblast differentiation and invasion. They may be required for appropriate activation of decidual iNKT cells at the maternal-fetal interface.

ICRI-07-1-37

Inhibition of human placental trophoblast proliferation by tumor necrosis factor-alpha is mediated by the induction of cytotoxicity, cell cycle arrest and apoptotic cell death

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Problem: Tumor necrosis factor (TNF)- α , a pro-inflammatory cytokine, expressed and produced at maternal-fetal interface, exerts both positive roles and negative regulator of pregnancy. Its interaction with human placental trophoblasts has been demonstrated in several studies, including the JAR trophoblast cell line. However, the mechanisms of inhibitory effect on JAR trophoblast cells have not been elucidated.

Method of study: The effects of this cytokine on cell proliferation and cytotoxicity were assessed by MTT and LDH assays, respectively. Production of hCG and progesterone was analyzed using electrochemiluminescence immunoassay. Cell cycle progression was investigated after restimulation of synchronized cells, using flow cytometric analysis. The induction of cell death was determined using RT-PCR for gene expression and DNA laddering and TUNEL techniques for DNA fragmentation.

Results: Recombinant human TNF- α treatment for 48 h resulted in a reduction of viable JAR cell number in a dose-dependent fashion, compared to the non-treated control groups ($p < 0.05$). Relative release of cytoplasmic LDH in TNF- α treated cells was markedly enhanced compared to the control groups ($p < 0.05$). In addition, these phenomena were paralleled with a marked reduction of hCG and progesterone productions. TNF- α induced cell cycle arrest in S phase resulting in delayed growth and is followed by cell death. Further analysis on the induction of apoptosis revealed that TNF- α treatment led to a down-regulation of bcl-2/bax expression ratio, and induction of apoptotic cell death.

Conclusions: Conclusively, these findings demonstrated that TNF- α , an immunodystrophism cytokine, regulates human trophoblast growth by the induction of cell cycle arrest and apoptotic cell death.

ICRI-07-1-38

Interleukin 1 in oviductal tissues of non-mammalian vertebrates

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Problem: Interleukin-1 (IL1) is a key regulator of the inflammatory response and an important mediator of implantation and embryo development in mice. We recently showed that IL1- β and its functional membrane receptor type I (IL1R1) are also expressed by reproductive tissues of non-mammalian vertebrates including squamate reptiles and elasmobranch fishes. Here, we investigated expression of IL1- β and its functional membrane receptor type I (IL1R1) in amphibians, a class of vertebrates characterized by different reproductive modes including internal and external fertilization.

Method of study: By immunohistochemistry using anti-human IL1- β and IL1R1 polyclonal antibodies we investigated the oviductal tissues of the aplacental viviparous *Salamandra lanzai*, the oviparous *Triturus carnifex* and the ovuliparous *Bufo bufo*.

Results: We demonstrated that in *S. lanzai*, most cells in the uterine mucosa were immunoreactive for IL1 β and IL1R1. In *T. carnifex*, IL1- β and IL1R1 were present in ciliated luminal cells, and there was evidence of IL1- β in glandular cells. In *B. bufo*, expression of IL1- β and IL1R1 was limited to the apical cytoplasm of the ciliated oviductal cells. Western blot analysis showed that a putative mature form of IL1- β , similar to that in mammals, was present in oviductal tissues of *S. lanzai* whereas different forms, likely corresponding to an inactive pro-IL1- β protein, were found in *T. carnifex* and *B. bufo*.

Conclusions: Although this study showed that IL1- β and IL1R1 expression occurred in all reproductive modes, the differential expression from ovuliparity to oviparity and viviparity may reflect the different

roles of IL1 system in the various reproductive modes.

ICRI-07-1-39

Theoretical basis for herbal medicines in the treatment of recurrent abortion: Tokishakuyaku-san and Sairei-to enhance the production of Th1 cytokines and GM-CSF in decidual stromal cells

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Problem: The herbal medicines such as Tokishakuyaku-san (Toki) and Sairei-to (Sai) have been empirically used in the treatment of recurrent abortion. However, there is little solid evidence on the pharmacological function of these medicines. Therefore, we investigated how Toki and Sai gave impact on the cytokine production from decidual mononuclear cells.

Method of study: Human DSCs were cultured with and without Toki or Sai at several different concentrations. The effect on cell proliferation was assessed by WST-8 assay. The amounts of cytokines release into culture media were examined by ELISA. Semi-quantitative PCR was carried out to see GM-CSF mRNA expression in DSCs. The cytokines assessed were interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-4, and granulocyte macrophage-colony stimulating factor (GM-CSF).

Results: Sai inhibited the proliferation of cultured DSCs, while no interference was observed in the presence of Toki. Both Toki and Sai enhanced the release of IFN- γ , TNF- α , and GM-CSF from DMCs, whereas no change was observed as to the release of IL-4. The increase of GM-CSF expression was as well confirmed at mRNA level.

Conclusions: Clinical benefit of Toki and Sai in the treatment of recurrent abortion might be attributable to the shift of Th1/Th2 polarisation and the enhancement of the cytokine supportive of pregnancy such as GM-CSF.

ICRI-07-1-40

Cytokine concentrations in women undergoing assisted reproduction technology as hypothetical marker of the success of ET

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Problem: It was determined that lack of tolerance of mother's immunity plays an important role in failure of pregnancy and that it is why the success rate of embryo transfer (ET) after in vitro fertilization (IVF) varies about 35%. We tested the hypothesis that the concentrations of cytokines (IL) 6, 10, 12, and tumor necrosis factor alpha (TNF α) in blood serum and follicular fluid influences the success rate of ET. Patients consist of women treated in our clinic for infertility using IVF-ET method. All women were stimulated using standard stimulatory protocols with rFSH and agonists or antagonists GnRH.

Method of study: Both blood and follicular fluid were taken in the day of ovum pick up. We tested sera and follicular fluids for the concentrations of cytokines using commercial ELISA kits (Immunotech). Statistical significance of the results was determined using non-parametrical t-test.

Results: Women were divided into two subgroups according to result of ET: successful (S-ET) and unsuccessful (U-ET). In the sera of S-ET, significantly higher concentration of IL-6 and IL-10 were found as compared with U-ET. TNF α was found in higher concentration in both sera and follicular fluids in S-ET, but the difference was not significant. IL-12 concentrations in follicular fluid were non-significantly higher in S-ET.

Conclusions: Our results indicate that the concentration of interleukins plays a role in success of ET and that the analysis of cytokines could bring valuable information for understanding immune mechanisms involving implantation of the embryo.

ICRI-07-1-41

Abnormal villous tree development in fetal growth restriction is accompanied by increased VEGF expression

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Problem: Fetal growth restriction (FGR) could reflect the abnormal placental villi formation. The aim of study was to perform the morphological characterization of villous vasculature and to measure concentrations of vascular growth factors in placentas of FGR newborns.

Method of study: Histological analysis was performed on paraffin sections of 70 placentas from full-term newborns with FGR and 71 placentas from full-term healthy newborns. VEGF and PlGF concentration in placental villi homogenates was determined by ELISA. Expression of VEGF and PlGF mRNAs in samples of basal plate and villous part of term placentas collected from 10 women with FGR and 12 normal pregnancies was measured by reverse transcription/real-time PCR.

Results: In 71.7% of FGR cases the villous tree was characterized by abnormalities of terminal villi varying from decreased number of syncytio-capillary membranes to terminal villi deficiency. Semi-quantitative morphometric analysis revealed a 5.8-fold decrease ($P < 0.001$) in villous vascularization in FGR. Expression of VEGF mRNA in villous part was significantly increased in FGR placentas as compared to normal ones (4-fold, $P < 0.01$). Furthermore, we observed elevated levels of VEGF protein in placental tissue homogenates in cases with terminal villi deficiency (252.5 ng/ml vs 34.3 ng/ml in normal mature placentas, $P < 0.001$). In contrast, neither mRNA nor the protein levels of PlGF were changed in FGR samples as compared to normal placentas.

Conclusions: We hypothesize that increased levels of VEGF in FGR placentas might shift a balance between branching and non-branching angiogenesis and thus lead to abnormal villous tree formation.

ICRI-07-1-42

Cytokines and chemokines in follicular fluid of women undergoing *in vitro* fertilization

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Problem: In vitro fertilization – embryo transfer (IVF-ET) technique is a potent tool for the treatment of fertility disorders (FD) but its success rate is limited by failure of implantation. Recent findings indicate that immunoregulatory cytokines control the induction of maternal immune tolerance to the embryo, and, consequently, the success of embryo implantation. The aim of our study was to determine the presence of selected cytokines and chemokines in follicular fluids (FF) of women undergoing IVF-ET.

Method of study: FF samples were collected as waste material after pick-up of oocytes. Two groups of patients were characterized as a) unexplained sterility with possible immune factor of FD (group A) and b) women with known non-immunological reason of FD (group B). We tested FFs for selected cytokines (IL-4, 6, 10, 12) by standard ELISA diagnostic kits or for complex cytokine and chemokine spectrum (23 cytokines and chemokines) by protein microarray analysis.

Results: Using ELISA, the group A of patients shown higher concentrations of IL-4 and lower concentrations of IL-6 and IL-10, as compared with group B. By microarray analysis, overall production was lower in group A where we observed mainly growth related oncogene (GRO), GRO-alpha, interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1. In group B following cytokines and chemokines were detectable: granulocyte and granulocyte-macrophage colony stimulating factor (G-CSF, GM-CSF), IL-1alpha, IL-2, IL-6, IL-7, IL-8, MCP-1 and MCP-2.

Conclusions: Chemokine and cytokine spectra of FF were different when we compared women with and without possible immunological factors of fertility disorders but further study is clearly necessary. This approach may be promising in the diagnostics of immunological factors involved in fertility disorders.

ICRI-07-1-43

Expression of CD1d and ligand-induced signaling are tissue-specific in mucosal epithelia of the human reproductive tract

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Problem: Mucosal epithelia of human reproductive tract are exposed to sexually transmitted microbes. CD1d, a lipid-antigen presenting molecule, plays a role in both innate and adaptive immune responses to microbes. The expression and function of CD1d in reproductive epithelial cells have never been studied.

Methods: Immunostaining for CD1d was performed on tissue sections of normal human reproductive tract. Differences in the expression of CD1d and in ligand-induced cytokine production were assessed using epithelial cells established from normal vagina, ectocervix, endocervix, and penile urethra. CD1d ligation was assessed by crosslinking using anti-CD1d 51.1.3 mAb. *Chlamydia trachomatis* infected to the penile urethral cells (MOI=1, 70% infection).

Results: CD1d immunoreactivity was strong in ectocervix and vagina whereas weak in endocervix and penile urethra. The expression in the penile urethral cells was weakest in the cells studied at mRNA and protein levels. Flow cytometry revealed that surface expression of CD1d was observed in ectocervical, vaginal, and penile urethral cells but not endocervical cells. IFN γ exposure induced CD1d strongly in vaginal cells. Ligation of surface CD1d induced IL-12 and IL-15 in ectocervical, vaginal, and penile urethral cells but not endocervical cells. Additionally, the CD1d-mediated cytokine induction in penile urethral cells was abrogated by *Chlamydia trachomatis* infection.

Conclusions: Expression and function of surface CD1d were strong in ectocervical, vaginal and penile urethral cells whereas absent in endocervical cells. Deficiencies in surface CD1d in the endocervix may result in their susceptibility to sexually transmitted agents. *Chlamydia trachomatis* downregulated the CD1d-mediated immune response to evade innate immune response.

ICRI-07-1-44

Expression of TLR2, 3, 4, and 9 genes in the human endometrium during the menstrual cycle

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Problem: Innate immunity in the endometrium has fundamental significance for reproduction. Although toll-like receptors (TLRs) play central roles in innate immune responses, their expression in the human endometrium remains to be fully elucidated. We examined the gene expression of TLR2, TLR3, TLR4, and TLR9 in endometrial tissues throughout menstrual cycle by quantitative PCR and in situ hybridization.

Method of study: Endometrial tissues were obtained from patients undergoing operations for benign gynecological conditions. Signed informed consent for use of the endometrial tissues was obtained from each woman. Specimen were dated according to the criteria of Noyes et. al. We examined expression of TLRs mRNA in endometrial tissues (N = 21) throughout the menstrual cycle by quantitative PCR. Secondly, we examined localization of TLRs mRNA in endometrial tissues (N = 18) throughout menstrual cycle. The intensity of staining in epithelial and stromal cells was separately evaluated by staining score.

Results: The expression levels of the four genes in the endometrial tissues varied in a similar pattern during the menstrual cycle; the levels were high in the perimenstrual period and low in the periovulatory period. The expression of the four genes was detected in both epithelial cells and stromal cells throughout the menstrual cycle. The expression levels were higher in epithelial cells in TLR3 and in stromal cells in TLR4, while they were comparable in epithelial and stromal cells in TLR2 and TLR9.

Conclusions: These findings imply that the differential spatiotemporal expression patterns of TLRs subserve a proper innate immunity of the endometrium.

ICRI-07-1-45

DNA microparticles delivered intranasally prime mucosal and systemic anti-SIV immune responses and protect against infection in rhesus macaques

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Problem: To study in rhesus macaques the systemic and mucosal immunogenicity of controlled release vaginal rings and biodegradable microspheres loaded with DNA encoding SIV proteins applied to different mucosal surfaces.

Methods: We prepared plasmid DNA, not replication competent, containing the whole SIVmac239 coding sequences driven by the CMV promoter (pcSIV). The construct was incorporated into poly(ethylene-co-vinyl acetate) (EVAc) matrices or poly(lactide-co-glycolide) (PLGA) microspheres. Three female rhesus macaques received vaginal rings loaded with 1 mg of pcSIV DNA and sutured in place (IVg). Three received 0.5 mg of pcSIV DNA loaded microspheres administered intra-nasally (IN). All animals were given intranasal booster immunizations of 0.5 mg DNA loaded microspheres at 6, 12 and 22 weeks, followed by 10⁷ plaque-forming units (PFU) vaccinia virus expressing SIV Gag, Env and Nef at week 28 IM. Two naive animals, were included as controls for primary immune response against VV. Animals were challenged intra-rectally (IR) with 1,000 TCID₅₀.

Results: Humoral responses against SIV were detectable after VV inoculation with DNA primed animals having stronger mucosal and systemic cellular responses than VV only. All naive animals became readily infected from the IR challenge with SIV. For the DNA/vaccinia animals, 4 of 6 were protected from viral infection.

Conclusions: Animals immunized by delivery of DNA in biodegradable microparticles to mucosal surfaces were protected from viral infection. While intranasal primary immunization resulted in stronger detectable immune responses, the challenge outcome was not different with one animal from each group becoming infected. Supported by NIH R01 EB00487.

ICRI-07-1-46

Studies of HIV-1 Virus Positive Discordant Couples in Kenya

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Problem: There are approximately 1.1 million HIV+ adults in Kenya. The availability of antiretroviral drugs and other lifestyle changes is increasing the lifespan of infected individuals.

Method of Study: We have identified HIV+ individuals from middle and upper income families whose partners have remained HIV negative. Fourteen DCs composed of 7 HIV+ Asian male partners and 7 HIV+ African partners (4 males, 3 females) were identified.

Results: From patient interviews, it was found that none of the couples used protection during intercourse. Seven of the DCs had been HIV+ for more than 9 years ("long term survivors"), three for 5 to 7 years and four for 2 to 3 years. Among the HIV+ partners, 9 had elevated CD8 cell levels at some point during the study and 13 had below normal CD4+ counts. There were fewer HIV+ women (3) in this DC cohort than men (11) with no HIV+ Asian females. All but three HIV+ individuals were Clinical Stage 1. These three progressed to Clinical Stage 2. To date, only one of the HIV- partners have seroconverted after 6 years. Two couples elected to have a child and, through counseling, both children were born HIV-.

Conclusions: These studies support earlier observations that prognostic accuracy may be improved by evaluating both CD4 and CD8 cell counts. The ethnic, genetic, social and financial status of the DCs may influence disease progression and infectivity. Further evaluation of such cohorts may provide a better insight into viral transmission and disease progression.

ICRI-07-1-47

MCMV - Induced Encephalitis and Developmental Brain Disorders in Newborn Mice

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Problem: Congenital human cytomegalovirus (HCMV) infection of the central nervous system (CNS) is the leading viral cause of mental retardation and progressive hearing loss. In order to study the pathogenesis of congenital HCMV infection within developing CNS we have developed a model of perinatal CMV infection of the CNS by infecting newborn mice with murine CMV (MCMV).

Method of study: Mice were infected intraperitoneally (ip) 6–12 h after birth then sacrificed at various days post infection (p.i.). Their brains were processed for immunohistochemical and Flow cytometry analysis.

Results: MCMV readily infects different cell types in developing mouse brain including neurons and glial cells. Parallel with visible infection, we observed deficits in postnatal cerebellar morphogenesis in terms of delayed granule neuron migration and disruptions in Purkinje cell morphology. We have also defined the phenotype of immune cells infiltrating the infected CNS. NK cells can be detected in the brain parenchyma within the first week p.i., while macrophages and T lymphocytes predominate from day 12 p.i.. CD8⁺ T lymphocytes represent the dominant immune population within infected CNS and MHC tetramer staining reveals that a significant amount of these cells is MCMV specific. Increased expression of CD28, a co-stimulatory molecule present on effector CD8⁺ T-cells, implies these cells have an activated phenotype.

Conclusions: Intraperitoneal MCMV infection of newborn mice results in a multi-focal encephalitis tied with delays in postnatal cerebellar development. The observed increase in mononuclear cell infiltration suggests that virus clearance is mediated by activated immune cells recruited from the periphery.

ICRI-07-1-48

Implantation in pigs is associated with quantitative changes and specific recruitment of immune cells in endometrium

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Problem: The role of endometrial immune cells in implantation and epitheliochorial placenta establishment.

Method of study: The quantity and pattern of distribution of CD45+, CD3+, CD4+, CD8+, γ/δ TCR, CD16+, CD56+, CD58, HLA-DR/DP+, CD45RD+, CD69+ cells in 10, 15, 20, 30 and 40 days pregnant endometrium, corresponding to the main implantation-related events; pre-receptive period, attachment and early post-attachment phases of implantation, definitive placenta and the end of early pregnancy were analyzed by flow cytometry and immunohistochemistry.

Results: Dominant endometrial lymphocytes were T cells with prevalence of Tc (CD3+CD8+) and NK (CD16+) cells. Total T, Tc, Th and activated cells were in highest number at the time of trophoblast attachment to the maternal epithelium, and NK cells significantly declined. γ/δ TCR, NK-like, CD56+, CD58+ and CD29+ cells present in relatively small and implantation uninfluenced proportions. *In situ*, T cells increased in amount with advance of implantation and formed T cell clusters (TCC) with implantation-stage dependent location. NK cells were in relatively low number during implantation and gradually increased after placenta establishment.

Conclusions: Implantation in pigs is associated with quantitative changes and recruitment of T cells from the deep endometrial regions toward the subepithelial stroma, and NK cells and B lymphocytes in the opposite direction – far from the attached conceptus. The work was partly supported by grant B 1537/05 from NFSI, Bulgarian Ministry of Education and Sciences.

ICRI-07-1-49

NK cell cytotoxicity is reduced by STAT3 silencing and sHLA-G

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Problem: Former studies indicated that HLA-G reduces availability of Signal Transducer and Activator of Transcription 3 (STAT3) in decidual NK cells and, thereby, perforin expression and cytotoxicity. Two different isoforms, STAT3-alpha and STAT3-beta exist, but their function in NK cells is not yet clear. The current study aims to further analyze the exact role of STAT3 in regulation of NK cell cytotoxicity.

Methods: STAT3 expression was silenced in the IL-2 dependent human cell line NK92 by applying two different small interfering RNA (siRNA) sequences. Silencing efficacy was measured by Western blots. NK92 cells were stimulated with IL-15, soluble HLA-G (sHLA-G) or both. Cytotoxicity to K562 cells was assessed by flow cytometry.

Results: Both sequences reduced STAT3 expression compared to native cells and cells treated with a non-genomic siRNA sequence, which had no influence on killing activity. One siRNA sequence had a stronger suppressive effect on the STAT3-alpha isoform than on STAT3-beta and reduced cytotoxicity more efficiently (between approx. 15 and 50 % depending on the different stimulation setups). The effects were strongest when cells were incubated with sHLA-G.

Conclusion: The results underline the cytotoxicity regulating role of STAT3 in NK cells and indicate an influence of the isoform balance.

ICRI-07-1-50

Natural killer and dendritic cell interaction at the maternal-fetal interface

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Problem: The aim of this study was to analyze the interaction of decidual natural killer (NK) cells with autologous mature or immature dendritic cells (DC).

Methods of study: Decidual mononuclear cells were isolated by enzymatic digestion and gradient density centrifugation. Phenotype, cytokine production and cytolytic mediator expression were measured by flow cytometry in NK and DC from the freshly isolated mononuclear cell suspension or after their purification and co-culture *in vitro*. Purification of CD56+ cells (>95%), immature CD1a+ (~35%) and mature CD83+ DC (~35%) was performed by positive magnetic separation. Proliferation of CFSE labeled CD56+ cells was analyzed by flow cytometer after the co-culture with mature and immature DC.

Results: The majority of decidual CD1a+ DC show immature phenotype with high expression of CD14 molecule. Less than 10% of the whole CD1a+ population express CD83 and show mature phenotype with higher expression of HLA-DR and co-stimulatory CD80 and CD86 molecules. The percentage of IL-15 and IFN- γ producing cells was higher in immature than mature DC subpopulations, whereas the difference in IL-10 and IL-18 production have not been observed. Immature DC enhance short and long term cytolytic mediators expression (18 hrs) and proliferation rate of NK cells (48 hrs), but decrease their pro-inflammatory (IL-15, IFN- γ TNF- α) cytokines production (18 hrs). Mature DC were inefficient in causing such effects. Both DC subpopulations support tolerogenic NK receptor repertoire expression on decidual NK cells.

Conclusion: It seems that both tissue specific DC subpopulations are able to shape NK cell functions at the maternal-fetal unit. The experiments are partially financed by Croatian Ministry of Science Grant

No. 0620402-0376 and EMBIC project, European FP6, NoE, No. 512040, LSHM-CT-2004-512040.

ICRI-07-1-51

IL-18 in the luminal environment before IVF/ICSI-ET: new insight for unexplained infertility

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Problem: Locally secreted cytokines control the implantation process and may be related in some cases to implantation failure. Expression of the interleukin-18 (IL-18) system in the uterine luminal environment was explored at the time of oocytes retrieval.

Methods: Uterine secretions of 274 patients enrolled in our IVF/ICSI-ET program were sampled by uterine flushings immediately before oocytes retrieval. 170 were endoluminal secretion alone (Group A) and 104 endoluminal secretion with endometrial cells (Group B). 23 patients were evaluated during two consecutive IVF/ICSI cycles. IL-18 expression was explored by Elisa assay and real time PCR if endometrial cells collected.

Results: IL-18 expression was significantly correlated during two distinct IVF/ICSI cycles ($r=0.54$, $p=0.009$). [IL-18] was significantly higher in Group B suggesting the endometrial secretion. Any significant differences were observed in function of either the protocols used or the outcome of the attempt. Implantation rate was significantly lower in group B compared to group A (14% versus 22% respectively). Regarding the etiology of infertility, IL-18 was significantly higher for patients with unexplained infertility.

Conclusion: IL-18 expression in the luminal environment at the time of oocytes retrieval is related to the patient itself and is not an indicator of the outcome.

ICRI-07-1-52

Regulation of granulysin protein expression in decidual NK cells by Th₁ cytokines and antigen presenting cells

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Problem: The aim of our study was to analyze the expression of a cytolytic protein granulysin in decidual CD56 positive cells after the stimulation with Th₁ cytokines and decidual antigen presenting cells.

Methods of study: Decidual mononuclear cells (DMC) were obtained by enzymatic digestion of decidual tissue and density gradient centrifugation. CD56+ cells and subsequently CD83+ cells were purified from the suspension of a non-adherent DMC fraction using positive magnetic separation after 18-hour culture at 37°C. The immature CD1a+ and subsequently CD14+ cells were isolated from the adherent DMC fraction. Granulysin expression was analyzed using cell permeabilisation method, flow cytometry, immunohistochemistry and confocal microscopy.

Results: Approximately 90% of decidual CD56+CD3- NK cells express granulysin in their cytoplasmic granules near the cell membrane. This percentage did not significantly change after 18-hour culture of NK cells in DMC suspension, nor after CD56 positive magnetic separation. The addition of anti-PIBF monoclonal antibody in the suspension of freshly isolated DMC decreased the percentage of granulysin expressing CD56+CD3- cells after 18-hour culture. The culture of purified CD56+ cells in the medium only for 18 hours, decreased the percentage of granulysin in their cytoplasm. This decrease is prevented by stimulation with IL-15, IL-2, and IL-18 in a dose-dependent manner. The co-culture of purified CD56+ cells with immature CD1a+ dendritic cells or CD14+ macrophages also upregulated the percentage of granulysin expressing NK cells, whereas CD83+ cells had no effect.

Conclusion: Decidual tissue specific environment, comprising PIBF, cytokines and antigen presenting cells, favors granulysin expression in CD56+ NK cells. The experiments are partially financed by

Croatian Ministry of Science (Grants No. 0620402-0376 and 0379) and EMBIC project, European FP6, NoE, LSHM-CT-2004-512040.

ICRI-07-1-53

Innate immune cells distribution throughout porcine implantation

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Problem: The amount and pattern of distribution of some innate immune cell populations – NK cells, eosinophils and macrophages in the peripheral blood and endometrium during implantation of pigs were studied.

Method of study: The study was performed in non-pregnant and pregnant sows on gestation day 10,15,20,30 and 40, corresponding to the main implantation-related events – pre-receptive period, attachment and early post-attachment phases of implantation, definitive placenta and the end of early pregnancy, respectively. Flow cytometry, immunohistochemistry, histochemistry and morphometry were used.

Results: NK cells (CD16⁺) number significantly declined in 15 days pregnant endometrium compared with non-pregnant in the same day of the cycle. NK-like lymphocytes (CD3⁺CD8⁺) and CD56⁺ cells were in comparable proportions in 15 days pregnant and non-pregnant endometrium. All NK-cell populations were in relatively low frequencies throughout the implantation and gradually increased after placenta establishment. The novel finding was the immunolocalization of NK cells in the porcine implantation and their implantation-stage influenced amount and pattern of distribution in situ. Eosinophils were in highest density in pre-receptive phase endometrium (gd 10). At attachment phase of implantation (gd 15) their infiltration dramatically declined. Macrophages in situ were in largest number prior to implantation (gd 10), dominating the subepithelial propria. With advance of implantation their number decreased and they were found to be equally distributed in both subepithelial and glandular stroma.

Conclusions: Diminished numbers and specific pattern of distribution of innate constituents of mater-

nal immune system (NK cells, eosinophils and macrophages) at materno-fetal interface during porcine implantation might be associated with noninvasive strategy for implantation and epitheliochorial type of placenta formation in this species.

ICRI-07-2-54

Human umbilical cord stroma cells (UCos) differentiate into endothelial cells

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Problem: Mesenchymal stem cells (MSCs) have the capability for renewal and differentiation into various lineages of mesenchymal tissue. MSCs are considered a strategy for repair of damaged tissues. The umbilical cord vein represents a promising source of MSCs. The aim of this study is to analyze the differentiation potential of UCos into cells of visceral mesodermal origin, such as endothelium.

Method of study: MSCs isolated from subendothelial layer of umbilical cord vein in accordance with established protocols. Fibroblast-like cells derived from umbilical cord vein were phenotypically characterized by flow cytometry, RT-PCR and immunofluorescence. Two types of differentiation were induced with these cells -osteogenic and endothelial.

Results: Cells isolated from subendothelial layer of umbilical cord vein displayed fibroblast-like morphology and grew forming colonies. Adherent fibroblast-like cells were negative by flow cytometry for hematopoietic line markers (CD45, CD14, CD3, CD19, CD16/56) and for HLA-DR and CD34 and were positive for CD29, CD90 and CD73. Analyzed cells were also positive for survivin, Bcl-2, vimentin and endoglin confirmed by RT-PCR and immunofluorescence. UCos cells were shown to differentiate in osteogenic cells. Further UCos cells were induced to differentiate into endothelial cells and this was proven by expression of CD31, vWF and KDR-2 and forming vessel-like structures in Matrigel.

Conclusions: The fact that umbilical cord stroma cells have the morphological and phenotypical characteristics of MSCs and are able to differentiate in different tissue types, makes them important and prospective source of MSC for use in cell therapy, tissue repairs and experimental needs.

ICRI-07-2-55

Isolation and characterization of multipotent stromal cells from human decidua

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Problem: Endometrium is considered to be a prospective source for isolation of multipotent adult stromal (MASC) cells because of the cyclic changes which are governed by neuroendocrine regulation. Presumably, MASC cells would take part in the formation of decidua during pregnancy. Adult stem cells are defined by their functional properties like high proliferative potential, clonogenicity and ability to differentiate into functional mature progeny. The aim of this study was to search for multipotent stromal cells present in decidual tissue.

Method of study: Samples were collected during surgery for extrauterine pregnancy after a signed informed consent. Decidual tissue was carefully dissected single-cell suspension was obtained using collagenase type IA. Cells were cultured in DMEM supplemented with 10% fetal bovine serum. Primary cell culture was characterized by flow cytometry using anti-CD45, CD34, HLA-DR, CD29, CD73 and CD90 antibodies (BD). To test for their clonogenicity cells were cultured at clonal density 200 and 500 cells/cm² in complete DMEM. In parallel experiments cells were induced to differentiate as osteogenic lineage following standard procedures.

Results: Growing cells showed typical fibroblast-like morphology, they were negative for haematopoietic markers and positive for CD29, CD73 and CD90 as shown by flow cytometry. The cultured cells could grow as singular colonies which seemed to be rather homogeneous morphologically. Specific accumulation

of Ca was observed in the intracellular space after induction to differentiate as osteogenic cells. The differentiated osteogenic cells were shown to express higher amounts of alkaline phosphatase which prove their capacity for osteogenic differentiation.

Conclusions: Our results demonstrated that human decidua contains multipotent stromal cells.

ICRI-07-2-56

Proportion of peripheral blood- and decidual- CD4⁺CD25^{bright} regulatory T cells and the expression of Toll like receptor 4 on interstitial extravillous trophoblast in pre-eclampsia

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Problem: CD4⁺CD25^{bright} regulatory T (Treg) cells have been identified as a principle regulator of tolerance during pregnancy, and persistent Toll-like receptor (TLR) signals inhibit Treg-mediated tolerance. In the setting of pre-eclampsia, however, little is known about the dynamics of these cells, or the interaction between Treg cells and TLR systems.

Method of study: We determined CD4⁺CD25^{bright} Treg cells in the peripheral blood using flow cytometry, and the expression of Foxp3, TLR4 and NFκB at the placental bed using immunohistochemical staining. Peripheral blood mononuclear cells (PBMC) of 38 pre-eclamptic cases (17 cases were Japanese, 21 cases were Polish), 40 normal late pregnancy subjects (20 subjects were Japanese, and 20 subjects were Polish), and 21 non pregnant healthy controls (10 subjects were Japanese, and 11 subjects were Polish) were included.

Results: We found the percentage of CD25^{bright} cells within the CD4⁺ T cell population in PBMC was significantly reduced in both Japanese and Polish pre-eclamptic cases than in normal pregnancy subjects (p<0.001) and non-pregnant healthy controls (p<0.001). Also, the percentage of Foxp3⁺ cells within CD3⁺ T cells in the placental bed biopsy samples of preeclamptic cases were decreased compared to those in normal pregnancy subjects. Interestingly, the expressions of TLR-4 and nuclear NFκB in interstitial extravillous trophoblast were increased

in pre-eclampsia, suggesting that the TLR pathway was activated at the placental bed.

Conclusions: These findings suggest that a decreased number of Treg cells and impaired function of Treg cells were present in preeclampsia, and these changes might break the maternal tolerance to the fetus.

ICRI-07-2-57

Antiphospholipid antibodies in mother and fetus in fetal-placental abnormalities

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Problem: APA have toxic effect on embryo and trophoblast and inhibit differentiation of myocyte and cytotrophoblast into syncytium in vitro. Poor embryo quality in APA positive IVF patients was confirmed. Research of association of APA in mother blood (MB) and fetal blood (FB) with placenta and embryo abnormalities was the aim of the study.

Method of study: Fetal blood from 30 high risk pregnancy women was received by cordocentesis on 20-23 weeks of gestation. Blood plasma was used for research after chromosomal analysis. FB and MB were analyzed for APA (IgG aCL, aPS, aPE), TNF, TNFR1, by ELISA. Research groups: 22 patients with abnormal placenta (AP) and 8 with normal development placenta (NP). In these women 16 fetuses have congenital anomalies (CAF) and 14 fetuses were normal (NF).

Results: APA frequency in MB of AP group was 48% 11/23 compared to NP patients 14% 1/7 ($p < 0.05$). MB APA levels in CAF and NF groups were the same. APA mean levels in FB were significantly higher when MB was positive for APA. But only in 2 FB from 11 positive mothers this level was significantly increased. In both cases phospholipid-specificity of APA in FB were the same as APA in MB and in them CAF and AP were confirmed. TNFR1 levels in FB were increased when APA were present in MB.

Conclusions: We did not find significant association between CAF in fetuses and APA. We show that APA are related with placenta abnormalities, and

may pass from mother to fetus (20-22 gw) resulting increase TNFR1 in fetus.

ICRI-07-2-58

IL-10 but not TGF- β is essential for the suppressor function of Treg cells in murine pregnancy

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Problem: The physiological state of pregnancy is characterised by the tolerance of the maternal immune system to paternal/fetal alloantigens. Regulatory T cells (Treg) were described to play an important role in the maintenance of this tolerance state and several mechanisms were proposed for their regulatory activity. In this context immune suppressive molecules such as IL-10 and TGF- β seem to be essential for Treg function. Therefore we aimed to investigate whether the pregnancy-protective effect of Treg is mediated by IL-10 or TGF- β .

Methods of the study: CBA/J females were mated with BALB/c males (normal pregnant). On day 14 of pregnancy Treg were isolated and transferred into DBA/2J-mated CBA/J females (abortion-prone). These animals were additionally treated with either anti IL-10, anti-TGF- β or IgG (isotype control) antibodies on days 0 and 7 of pregnancy. Normal pregnant and untreated abortion-prone females were used as controls.

Results: Treg + IgG-treated animals had a similar abortion rate as the normal pregnant group. In contrast CBA/J females which received Treg and were additionally treated with anti-IL-10 presented an increased abortion rate, which was even higher than in the untreated abortion-prone group. The application of anti-TGF- β did not substantially modify the abortion rate as compared to Treg + IgG-treated animals.

Conclusions: These results suggest that IL-10, but not TGF- β is essential for the immune regulatory function of Treg during pregnancy. It further underlines the assumption that Treg mediate their suppressive activity against alloreactive maternal immune

cells through the release of immune suppressive molecules *in vivo*, being IL-10 essential in this context.

ICRI-07-2-59

Protective effect of Treg during murine pregnancy depends on Heme Oxygenase -1

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Problem: Regulatory T cells (Treg) play an important role in fetal protection. It is known that Treg expand during normal pregnancy and protect paternal/fetal antigens from maternal effector cells. The transfer of Treg obtained from BALB/c-mated CBA/J females has been shown to prevent abortion in DBA/2J-mated animals. This was shown to be associated with the up-regulation of so-called tolerance associated molecules at the fetal-maternal interface, namely Heme Oxygenase (HO-1), Leukaemia inhibitory factor (LIF) and Transforming growth factor-beta (TGF- β). Among these, HO-1 is an interesting candidate as its blockage or absence correlates with increased abortion rates or even infertility. Therefore, we aim to investigate whether the protective effect of Treg is mediated by HO-1 in a mouse model.

Method of study: To block HO-1 in animals rescued from abortion after Treg transfer on day 1 (CBA/J x DBA/2J + Treg), treatment with Zn-PP on days 0, 3 and 6 of pregnancy was done. Abortion-prone (CBA/J x DBA/2J) or normal pregnant animals (CBA/J x BALB/c) treated with either PBS or Zn-PP were included as controls.

Results: As expected, Treg-treated animals had no abortion. This protective effect of Treg was abrogated when HO-1 was blocked by means of Zn-PP. Furthermore, the application of Zn-PP to normal pregnant and abortion-prone animals resulted in increased abortion rates.

Conclusions: Our data clearly show that the protective effect of Treg is not achieved when HO-1 is blocked strongly suggesting that Treg are protective through up-regulating HO-1 further bringing to light one of the several pathways through which Treg mediate tolerance towards the semi-allogeneic fetus.

ICRI-07-2-60

Phenotypic characterization of peripheral blood - and decidual naturally occurring regulatory T cells from human normal early pregnancy

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Problem: The natural regulatory T (Treg) cells are enriched during gestation and are proven to be of great importance for the successful outcome of pregnancy. The objective of this study was to investigate and compare the phenotype of natural CD4⁺CD25⁺ Tregs in peripheral blood and decidua from human normal early pregnancy.

Method of study: CD4⁺CD25⁺ cells were extracted and enriched from paired peripheral blood- and decidual samples and characterized using immunohistochemistry, immunoflow cytometry, immunofluorescence and quantitative RT-PCR.

Results: CD4⁺CD25⁺ Treg cells are constitutive inhabitants of human early pregnancy decidua. Two subpopulations of decidual natural Tregs expressing FOXP3 were identified - CD4⁺CD25⁺FOXP3⁺ and CD4⁺CD25⁺FOXP3⁻. Interestingly, approximately 10% of the CD4⁺CD25⁻ decidual lymphocytes expressed FOXP3 as well. All three FOXP3⁺ cell subsets were significantly enriched in decidua compared to peripheral blood as shown by analyses of paired samples. The decidual CD4⁺CD25⁺FOXP3⁺ cells further expressed the activation markers CTLA4, neuropilin, LAG-3 and CD45RO and mRNA for TGF β 1.

Conclusions: Here we report for the first time that a subset of CD4⁺CD25⁻ cells in human decidua expresses FOXP3 and demonstrate detection of CD4⁺FOXP3⁺ Tregs *in situ*. A similar unusual CD4⁺CD25⁻FOXP3⁺ population was recently described in mice and proposed to be a naïve Treg phenotype. Thus, our results suggest three main FOXP3⁺ Treg subsets in human decidua - CD4⁺CD25⁺FOXP3⁺, CD4⁺CD25⁺FOXP3⁻ and CD4⁺CD25⁻FOXP3⁺. Further experiments for elucidation of their function are in progress. All three FOXP3 expressing Treg subpopulations are actively enriched in human decidua compared to peripheral blood and might be associated with maintenance of the maternal immune tolerance to the fetus.

ICRI-07-2-61

Regulatory T cells in Recurrent spontaneous abortion

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Problem: It is a well-known observation that during pregnancy the mother's immune system is under serious control. Some elements of this regulation has already been revealed mainly the ones occur locally at the placenta. Behind miscarriage several disorders can hide. One of them is an immunological problem from unexplained reason. The treatment of this subgroup of recurrent spontaneous abortion (RSA) can highly be supported by administration of high dose of intravenous immunoglobulin (IVIG) therapy and/or thrombocyte transfusion (TF) prior conceiving. The involvement of T regulatory (Treg) cell in pregnancy and their decrease in RSA has been shown. We focused on this form of RSA, and followed the numbers of Treg cells before and after treatment extending the follow up during pregnancy, as well.

Method of study: We compared Treg cell number in normal pregnancy, untreated RSA suspected cases at whom the unexplained immunological background was conformed later and treated RSA subjects using Fluorescence-activated cell sorting (FACS) technology by tricolor labeling with anti-CD4/anti-CD25/anti-CD127 from peripheral blood.

Results: Full series of TF increases the Treg number to the level of normal pregnancy. Partial TF has no or slight increasing effect. IVIG transfusion leaves the number of Treg cells unchanged.

Conclusion: TF acts as an "active immunization" while IVIG administration does not change the level of these cells in a "passive immunization" way.

ICRI-07-2-62

Effects of anti β 2-GPI antibody positive sera on PlGF production from cultured choriocarcinoma cell line

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Problem: The anti β 2-GPI antibody was detected in recurrent abortion and intrauterine death. Placenta Growth Factor (PlGF) is a growth factor to accelerate villous proliferation. We evaluate the effects of anti β 2-GPI antibody positive sera on PlGF production from cultured choriocarcinoma cell line and cytotoxicity.

Method of study: Choriocarcinoma cells (JEG-3) were cultured with 24 well plates. Each serum was added to the culture medium of choriocarcinoma cell at the concentration of 10%. The PlGF in culture medium was measured by ELISA (R&D) 24 hours after. The survival rate of cells was measured by the XTT method. The serum were taken from 6 anti β 2-GPI antibody positive and 6 negative patients and those were sterilized with Millipore filter.

Results: The concentration of PlGF in supernatant by anti β 2-GPI antibody positive sera was significantly lower than that of anti β 2-GPI antibody negative. There was no significant difference of the values of XTT by each serum between them.

Conclusions: The anti β 2-GPI antibody positive sera suppress the production of PlGF from choriocarcinoma cell. We suggested that the anti β 2-GPI antibody may suppress PlGF production from trophoblast and cause the failure of placenta formation and function.

ICRI-07-2-63

Mast-cell-associated genes *Tph1*, *Mcpt1* and *Mcpt5* are up-regulated after Treg-induced tolerance at the fetal-maternal interface: new role for mast cells in tolerance?

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Problem: CD4⁺ CD25⁺ foxp3⁺ regulatory T (Treg) are known to play a major role in murine pregnancy outcome by inducing tolerance towards the semiallogenic fetus. Mast cells (MC) are known as primary responders in allergic reactions. However, recent studies show that MC play a critical role in the Treg-dependent allograft tolerance by secreting interleukin-9 (IL)-9. Here, we investigated whether MC may be also involved in Treg-mediated tolerance of the fetus.

Method of study: The mRNA expression of MC-associated genes (tryptophan hydroxylase (*Tph*)-1 as well as mast cell protease (*Mcpt*)-1 and *Mcpt*-5 in normal pregnant (NP) mice, abortion-prone (AP) and abortion-prone mice treated with Treg (AP+Treg) was analyzed in placental samples by real-time RT-PCR.

Results: The expression of *Tph1*, *Mcpt*-1 and *Mcpt*-5 was comparable at early pregnancy stages between NP and AP mice. Interestingly, the expression of all three molecules was down-regulated on day 14 of pregnancy in placenta samples from AP compared to NP mice. Moreover, Treg treatment markedly up-regulated their expression to the levels of NP mice.

Conclusions: These results indicate that as already observed in transplantation, mast cells-associated molecules might contribute to Treg-induced tolerance at the fetal-maternal interface.

ICRI-07-2-64

Heme oxygenase-1 (HO-1) may be involved in follicle development and fertility

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Problem: The pathways underlying immunologic tolerance at the fetal-maternal interface are still not fully characterized. Previous studies suggest that Heme Oxygenase-1 (HO-1) may be essential for successful pregnancy. Further, no progeny is achieved when mating mice where the *Hmox-1* gene has been deleted. We aim to clarify whether this is due to sterility or lack of implantation, by investigating whether HO-1 deficient females are unable to produce oocytes capable to develop into blastocysts, or if the viable blastocysts are unable to implant due to uterine inadequate conditions, respectively.

Method of study: We injected wild type (wt) and *Hmox-1*^{-/-} females with PMSG/hCG in order to induce superovulation and fertilized these oocytes by *in vitro* fertilization.

Results: *Hmox-1*^{-/-} females (n=8) produce much less oocytes than wt females (n=8). Analyses of the ovaries of both groups showed differences in morphology and in follicle development. Furthermore, wt oocytes fertilized with wt sperm showed a fertilization rate of 61,70%, while *Hmox-1*^{-/-} females fertilized with *Hmox-1*^{-/-} sperm showed a fertilization rate of 19,78%.

Conclusions: These results suggest that HO-1 may be necessary but not indispensable for fertilization. Besides, *Hmox-1*^{-/-} oocytes are not as viable as wt oocytes, which may be the reason why the mating of mice heterozygous for HO-1 yield so few *Hmox-1*^{-/-} progeny, and why *Hmox-1*^{-/-} animals do not success to get pregnant when paired with *Hmox-1*^{-/-} males. These results do not exclude the important role of HO-1 in implantation, but suggest that the lack of HO-1 may also affect fertility.

ICRI-07-2-65

The level of potentially immune-affecting free amino acids in preeclampsia

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Problem: Preeclampsia one of the most common diseases in pregnancy, is associated with abnormal immune system function. Amino acids are not only the structural units of proteins, but also have immunological properties. Of particular interest are the amino acids: glutamine, glycine, taurine, tryptophan and arginine. Glutamine is the most important source of energy for macrophages and lymphocyte to proliferation, antigen presentation, cytokine production and phagocytosis. Glycine, another nonessential amino acid, exerts modulatory effects on cytokine production, by inhibiting activation of neutrophils. Tryptophan and taurine, play important role in host defense and antioxidant protection. Enhanced tryptophan degradation during pregnancy via activation of indoleamine-2,3-dioxygenase, was found to be necessary to achieve immunotolerance against the fetus. Arginine plays a role in the regulation of NO synthesis. The aim of the study was to examine the relationships between the availability of plasma free amino acids and preeclampsia.

Method of study: Total plasma concentrations of glutamine, glycine, taurine, tryptophan, and arginine were measured in 20 healthy pregnancies, and 20 women with preeclampsia. Plasma amino acids were measured by ion exchange chromatography on automated amino acid analyzer.

Results: Women with preeclampsia exhibited significantly higher glutamine and tryptophan plasma concentrations than did normal pregnant women, whereas glycine and taurine levels were significantly lower in women with preeclampsia compared with those in pregnant controls. No differences were found in arginine between study groups.

Conclusions: The results of this study demonstrate that availability of free amino acids in preeclampsia may contribute to the pathogenesis of this complex disorder.

ICRI-07-2-66

The level of plasma arginine-family free amino acids in preterm labor

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Problem: Many various social, nutritional and pathological factors are related in pathogenesis of preterm labor. Nitric oxide plays an important role in regulating placental-fetal blood flows and uterine contraction. Amino acid L-arginine is a substrate for NO and L-citrulline syntheses via NO-synthase (NOS). In vitro study indicates that another amino acid-glutamine can limit arginine availability for NO production by inhibiting recycling of citrulline to arginine. The aim of this study was to compare maternal plasma free amino acids levels between normal pregnancies and those with preterm labor, in order to determine whether maternal arginine-family amino acids deficiency are implicated, due to lower NO synthesis, in preterm delivery.

Method of study: The mean plasma concentrations of arginine, its precursor ornithine citrulline and its precursor glutamine, and ratio of L-citrulline to L-arginine, which reflects NO production, were measured in maternal blood samples from pregnancies between 32 and 39 weeks gestation. Plasma amino acids were measured by ion exchange chromatography on automated amino acid analyzer. Results are expressed as mean±SEM. Values of p<0.05 were considered to be statistically significant.

Results: Our results show that maternal plasma glutamine and citrulline concentrations in preterm labor were significantly lower than those in term pregnancies. There were no significant differences in plasma concentrations of ornithine and arginine between the two study groups. Cit/Arg ratio, which reflects NO production, was lower in pregnancy associated with preterm labor.

Conclusions: Our results confirm previous observations that decreased nitric oxide production may be important in the pathophysiology of preterm delivery.

ICRI-07-2-67

Characterization of novel Treg-mediated mechanisms of tolerance at the fetomaternal interface

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Problem: Normal murine pregnancy (NP) is associated with an expansion in the CD4+CD25+foxp3+ regulatory T cell (Treg) subset. Additionally, the adoptive transfer of Treg from NP into abortion-prone animals (AP) can rescue these mice from abortion. The mechanisms by which Treg act locally remain not fully elucidated.

Method of study: mRNA microarray analysis was employed to compare the gene profile of AP mice and NP mice. To investigate which molecules were further modified after Treg transfer, we compared the gene profile between Treg-treated animals and AP animals.

Results: When comparing AP vs NP mice we found 8 genes differentially expressed in decidua and 42 in placenta. Interestingly, the Treg treatment in AP mice resulted in the modification (up- or down-regulation) of 97 genes in decidua and 87 genes in placenta. From these molecules, we concentrated in three from which we confirmed their modulation after Treg treatment by real time RT-PCR: Prostaglandin F2 α Inhibin A and Urokinase-type plasminogen activator (uPA).

Conclusions: Our data show that molecules not directly related with immunological pathways were differentially regulated after Treg treatment as Prostaglandin F2 α is responsible for uterine contractions, inhibin A was shown to be augmented in pre-eclampsia and uPA is released by the extravillous trophoblasts. These data indicate that Treg act not only by restoring the levels of molecules differentially expressed in AP to the levels observed in NP mice but also by modifying other molecules locally. We uncovered novel molecules associated with Treg-induced tolerance, which are worthy of further functional studies.

ICRI-07-2-68

Indoleamine 2,3-dioxygenase mRNA expression in different tissues of neonatal mouse during *Listeria monocytogenes* infection

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Problem: *L. monocytogenes* infection causes serious morbidity during the fetal and neonatal period. Bacterium has a predilection for placenta and brain tissue. Previous studies have shown the involvement of the indoleamine 2,3-dioxygenase (IDO) in maintaining the immune privilege of these organs. In this study we investigate the role of TNF receptor 1 (TNFR1) in IDO regulation during experimental neonatal listeriosis.

Method of study: We infected wild-type (WT) C57BL/6 and TNFR1 knockout (KO) newborn mice with attenuated actA-deficient *L. monocytogenes*. Tissue levels of IDO mRNA were detected by RT-PCR. The extent of cell infiltration in the organs was assessed using immunohistochemical methods. The number of bacteria in tissue was determined using conventional microbiological techniques.

Results: Infection resulted in a hyperinflammatory and ultimately fatal host reaction and high bacterial load in KO mice in contrast to 100% survival rate without clinical disease in WT mice. The basal levels of IDO in WT and KO mice were the highest in the brain tissue. After infection basal levels of IDO mRNA transcription in liver, spleen and brain of WT mice were retained while in KO mice were enhanced.

Conclusions: Macrophage tissue infiltration was paralleled by an induction of IDO mRNA in *L. monocytogenes* infected KO mice. KO mice succumbed to uncontrolled infection and inflammation mediated injury, despite high local IDO mRNA expression. TNFR1 is required for the efficient antilisterial host immune response.

ICRI-07-2-69

Signal Transducer and Activator of Transcription 3 (STAT3) in Jeg-3 choriocarcinoma cell lines: regulation of its serine and tyrosine phosphorylation balance

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Background: Signal Transducer and Activator of Transcription 3 (STAT3) is an intracellular signalling molecule, which is used by a wide spectrum of cytokines. It induces a variety of gene transcripts and cell functions. In trophoblast cells and in tumor cells, its tyrosine phosphorylation is directly linked to their invasiveness. The regulation and function of STAT3 serine phosphorylation is still unclear.

Material and Methods: Jeg-3 choriocarcinoma cells were stimulated with different concentrations of EGF, HGF, IL-4, IL-6, LIF, IL-15, IGF-II, IFN- γ and GM-CSF. STAT3 serine (727) and tyrosine (705) phosphorylation were analyzed 5 to 60 minutes after stimulation by SDS-PAGE electrophoresis followed by Western blotting.

Results: Jeg-3 cells display spontaneous STAT3 serine phosphorylation. 100ng/ml EGF induces a time-dependent reduction starting 15 minutes after stimulation. Tyrosine phosphorylation does not occur spontaneously, but is strongly induced by EGF at all analyzed time points. LIF induces tyrosine phosphorylation, but affects serine phosphorylation only very slightly. HGF and IL-15 induce and IL-4 reduces serine phosphorylation slightly. IGF-II, IFN- γ , GM-CSF and IL-6 did not remarkably influence neither serine phosphorylation nor tyrosine phosphorylation.

Discussion: The results demonstrate that serine/tyrosine phosphorylation balance of STAT3 is controlled by several cytokines. The combination with the STAT3-alpha/beta balance and the STAT3/SOCS3 balance seems to regulate choriocarcinoma and trophoblast functions crucially.

ICRI-07-2-70

Ras-signaling in trophoblastic cells

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Problem: Numerous cytokines influence functions and behaviour of trophoblast cells. The use of different signalling pathways is decisive for different functions, such as migration, proliferation and invasion. Epithelial Growth Factor (EGF), Leukemia Inhibitory Factor (LIF) and Interleukin-6 (IL-6) are major inducers. MAP Kinase (MAPK) pathways, which are related to invasiveness in many malignant cells, are expected to be involved.

Material and Methods: Jeg-3 choriocarcinoma cells and the Jeg-3-human trophoblast hybridized cell lines ACH-3P (first trimester) and AC1-M59 (third trimester) were stimulated with EGF, LIF or IL-6. After 2 to 30 minutes, cells were lysed and activated (GTP-bound) ras was pulled down by using small modular GTPase-binding domains derived from c-Raf and sepharose micro-beads. The ras-containing pellet was analyzed by electrophoresis followed by Western blotting and detected with various anti-ras antibodies. The remaining lysate was analyzed for phospho-erk1/2.

Results: EGF induces a strong and immediate ras and erk1/2 activation visible at all analyzed time points in trophoblast hybridized cell lines. In ACH-3P also LIF and IL-6 induced such activation but at lower level. In AC1-M59 effects of LIF started with a delay of 15 minutes after stimulation. IL-6 did not induce detectable effects. Jeg-3 cells displayed a high spontaneous ras activation which was not further increased after stimulation.

Conclusion: The observed differences indicate different intracellular regulation mechanisms in trophoblast cells derived from different trimesters and in choriocarcinoma cells. Especially LIF induced ras signaling seems to be ambivalent in early and late pregnancy. The high spontaneous ras activation in Jeg-3 cells may be related to their malign characteristics.

ICRI-07-2-71

Invasive or not? The STAT3 - SOCS3 balance regulates trophoblast and choriocarcinoma behaviour

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Problem: Invasion of human trophoblast cells requires a fine tuning, which is fundamental for correct placentation. Similar to tumor invasion, Signal Transducer and Activator of Transcription (STAT)3 is involved in this process. Its negative regulation is crucial to avoid invasion excess in human trophoblast cells and may be important to maintain non-invasiveness in other species (e.g. sheep). This study aims to analyze the role of Suppressor of Cytokine Signaling (SOCS)3 for the control of invasion of human choriocarcinoma cells used as a model for trophoblast cells.

Methods of Study: Small interfering RNA (siRNA) for SOCS3 silencing was validated on human Jar choriocarcinoma cells and served to self-design vector based small hairpin RNA (shRNA), which was integrated into pSuper vector, multiplied by E.coli and transfected into Jar cells. Based on neomycin resistance a stable SOCS3 silenced cell line was established. After stimulation with LIF, proliferation, invasion as well as activation of STAT3 signaling was measured by using flow cytometry, matrigel assays, and Western blotting, respectively.

Results: In SOCS3 silenced cells LIF induced proliferation, matrigel invasion and STAT3 phosphorylation was increased. This effect was stronger when SOCS3 silencing was performed by shRNA than by siRNA. Further, unstimulated SOCS3 shRNA silenced cells displayed elevated spontaneous invasion and STAT3 phosphorylation.

Conclusion: Activation of STAT3 and expression of SOCS3 regulate proliferative and invasive capacities of JAR choriocarcinoma cells, which may be regarded as a model for trophoblast cells. The balance of SOCS3 and STAT3 signaling seems to be a cross species key regulator in trophoblast and tumor cell invasion.

ICRI-07-2-72

Rapamycin induces mTOR independent PI3K signaling increases invasiveness of trophoblast-like cells

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Introduction: Mammalian target of rapamycin (mTOR) is an intracellular key regulator for cell growth and proliferation, involved in hepatocyte growth factor (HGF) signalling. Rapamycin is an inhibitor of mTOR kinase activity. The mechanisms of regulation of trophoblast invasion by various signal cascades are poorly understood, and are widely similar to but its unraveling may indicate relevance to tumor growth.

Material/Methods: The choriocarcinoma cell lines JEG-3, JAR and choriocarcinoma-trophoblast hybrid cell lines AC-1M59 and ACH3P were treated with rapamycin for 48h and analyzed for proliferation (MTT), apoptosis (cleaved PARP), MMP2 expression (Western blot), MMP2/9 activity (zymography) and migration/ invasion (matrigel-coated inserts). Phosphorylation of mTOR kinase substrate P70 S6K and in parallel of P85 S6K, the regulatory subunit of PI3K were analyzed.

Results: The upregulation of P85 S6K phosphorylation, was concomitantly found with downregulation of P70 S6K phosphorylation when cultured with rapamycin. Treatment with rapamycin increased migration and invasion whereas proliferation was reduced in all cell lines. Strikingly, apoptosis rate was reduced by rapamycin. There was no clear change in MMP2 expression/ activity.

Conclusion: The potential of rapamycin to inhibit mTOR activity accompanied with activation of Akt, a signal molecule upstream of mTOR and downstream of PI3K, resulting in pro-survival signals has already been reported. However, so far no negative feedback loop is described in which Akt regulates PI3K. We thereby suggest that there may be a novel regulatory feedback mechanism, either between Akt and PI3K or mTOR and PI3K, which accounts for the unexpected stimulation of invasion and anti-apoptotic behaviour upon rapamycin treatment.

ICRI-07-2-73

Immune cell profiling in normal pregnancy, partial and complete molar pregnancy

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Problem: Cytotoxic T cells (Tc) and natural killer (NK) cells may play a pivotal role in controlling trophoblast invasion. Regulatory T cells (Treg) are known to suppress the cytotoxic immune response *in vitro*. The role of Treg and the activation level of cytotoxic immune cells in gestational tissue remains unclear.

Method of study: Immunolocalization of CD8, Granzyme B (GrB), and FoxP3 was performed on sections prepared from eleven gestational age-matched normal placentas, nineteen partial moles (PM), and eighteen complete moles (CM) to characterize active (GrB+CD8+) and inactive (GrB-CD8+) Tc cells, active NK cells (GrB+CD8-), and Treg (FoxP3+) cells.

Results: Immune cells infiltrated into the implantation site of normal placenta, PM, and CM with increasing frequency. Active and inactive Tc, active NK and Treg infiltration in the CM were significantly stronger than seen in the normal placenta ($p=0.002$, $p=0.002$, respectively). Additionally, immune cell infiltration was not detected in the villi or trophoblast of gestational tissues. Treg infiltration in the implantation site was only observed in PM and CM. In CM and PM Tc infiltration positively correlated with Treg infiltration ($p=0.001$), but Treg infiltration did not correlate with the Tc activation ratio (active Tc cells / all Tc cells).

Conclusion: In CM the cellular immune response in the implantation site was significantly more vigorous than seen in normal placenta. These observations demonstrate that in the implantation site of CM, the number of active Tc and the activation of the NK cells increase and Treg cells may negatively regulate T lymphocyte activation.

ICRI-07-2-74

Type 1 versus type 2 immune response balance of decidual innate and adaptive cells during first trimester of pregnancy

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Problem: Previous studies have reported the importance of a bias of T cell immunity towards type 2 (Th2) for normal pregnancy outcome. However, some recent studies performed on peripheral blood and decidual lymphocytes showed also relevance of Th1 responses in maintenance of normal pregnancy. To address this problem we studied the expression of IL18Ralpha (which is considered to be a functional marker of type 1 responses) and ST2L (a homologue of IL1R, which is a marker of type 2 responses) on decidual lymphocytes

Methods of study: Decidual lymphocytes were isolated by ficoll gradient centrifugation of the enzymatically digested decidual tissue obtained from normal first trimester pregnancy. Expression of IL18Ralpha and ST2L markers were detected on the freshly prepared decidual T-cell subpopulations; (CD3+CD4+; CD3+CD8+), NK cells (CD56^{bright}CD3-; CD56^{dim}CD3-) and NKT cells (CD56+CD3+) after three-colour immunofluorescence staining with directly and indirectly conjugated antibodies followed by flow cytometry.

Results: High expression of IL18Ralpha and low expression of ST2L was detected on all investigated cell populations. Almost 60% of CD3+CD4+ cells express IL18Ralpha, and less than 2% ST2L (Th1/Th2 ratio was 46,0). Approximately a half of CD3+CD8+ T cells express IL18Ralpha while only less of 2% ST2L (Tc1/Tc2 ratio was 39,6). Similar results were obtained on NK cells, but the NK1/NK2 ratio was lower suggesting more of a bias to type 2 than in the T cell populations. Decidual NK cells (CD56+CD3-) and NKT cells (CD56+CD3+) also express predominantly IL18Ralpha (13% and 66,3% respectively) in comparison to ST2L (1% and 1,5% respectively). We found stronger IL18R expression on CD56^{dim}CD3- cells than on the CD56^{bright}CD3- (24,9% and 4,1%, respectively), but calculated NK1/

NK2 ratios for both populations remain very similar, for CD56^{bright}CD3- population ratio was 19,1 and for CD56^{dim}CD3- population was 17,2. In contrast, the NKT1/NKT2 ratio was very high (53,5).

Conclusion: High expression of the IL18Ralpha than ST2L on decidual cells of innate and adaptive immunity indicates importance of the type 1 immune response for normal pregnancy maintenance. The experiments are partially financed by Croatian Ministry of Science (Grants No. 0620402-0376 and 0379) and EMBIC project, European FP6, NoE, LSHM-CT-2004-512040.

ICRI-07-2-75

The seventh complement component is expressed on decidual endothelial cells membrane and exerts an anti-inflammatory action

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Problem: The seventh complement component (C7) is essential for the assembly of the terminal complex (TCC) that has cytotoxic and inflammatory activities. We previously showed that endothelial cells (ECs) synthesize this component. We now evaluate the presence and the role of C7 on the membrane of ECs from umbilical veins and human decidua.

Method of study: We evaluated the presence of membrane bound C7 on the ECs using IHC, IF, TEM and ELISA on whole cells. The function of C7 was determined by following the expression of adhesion molecules by ELISA on whole cell, quantifying the IL-8 production by ELISA, and evaluating the effect on ECs permeability by a Transwell system.

Results: The IHC study on frozen sections revealed the presence of C7 on EC from human umbilical vein and decidua. This data were also confirmed evaluating the membrane bound C7 on isolated ECs. Membrane C7 maintain the ability of soluble C7 to bind the other terminal components leading to the assembly of the TCC on the ECs membrane. The

membrane bound complex inhibits the expression of adhesion molecules that it is stimulated by soluble TCC, blocks the secretion of IL-8, and abrogates the increased albumin leakage induced by soluble TCC.

Conclusions: The TCC assembled on membrane C7, unlike the soluble TCC, does not exert any proinflammatory activity, on the contrary it offers to the ECs protection against the inflammatory effect and it is very important in tissues where the inflammation could cause damage as in decidua.

ICRI-07-2-76

Endoplasmic reticulum-resident heat shock protein gp96, natural killer T and regulatory T cells as regulators of self-tissue damages induced by various types of injuries

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Problem: Various kinds of tissue disintegration lead in the endoplasmic reticulum (ER) to activation of adaptive pathways known as the ER stress response, which is directed to correction of unfolded proteins, to the activation of proteasome-dependent ER-associated degradation of the misfolded proteins and to activation of protein translation that modulate the polypeptide traffic into the ER. A crucial role in these events plays gp96, which acts not only as a molecular chaperone but also as an adjuvant, able to induce the specific immune response against some self and foreign antigens.

Method of study: Since in the protection from the development of dangerous autoimmunity a special function might have self-reactive NKT cells and regulatory T cells (Treg) in this study we analysed the interplay between the gp96, NKT and Treg in conditions of: 1) normal growth, induced by partial hepatectomy, 2) in psychosocial stress without the tissue lesions and 3) after the treatment with bacterial peptidoglycan-monomer linked with zinc. Tissue expression of gp96 protein and mRNA was estimated in liver, thymus and spleen and the data were correlated with phenotype and cytotoxicity of hepatic and splenic mononuclear lymphatic cells (MNL) against the syngeneic thymocytes, NK and LAK-sensitive targets.

Results: All procedures induced fast cytoplasmic overexpression of gp96 staining in hepatocytes, followed by surface expression of gp96 on MNLc and upregulation of gp96 mRNA in the spleen and in the thymus. Simultaneously, in the liver accumulated CD3^{intermediate}/NK1.1⁺/CD69⁺ cells, as well as Treg cells, while hepatic (after initial suppression) and splenic MNLc became highly cytotoxic against syngeneic thymocytes and YAC-1 and P815.

Conclusions: The data imply that during the disturbance of morphostasis gp96 may serve as a natural adjuvant for chaperoning antigenic self peptides into the immune surveillance pathways, resulting in activation of autoreactive NKT which in cooperation with Treg cells participate in reestablishment of self tolerance. Supported by Croatian Ministry of Science, Grant No. 0621341-1337.

ICRI-07-2-77

The expression and possible roles of chemokine CXCL11 and its receptor CXCR3 in the human endometrium

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Problem: IFN- γ secreted by a human embryo and trophoblast cells during implantation is suggested to play an important role in implantation and pregnancy. IFN- γ is known to strongly induce CXCL11, a CXC chemokines, in a range of cell types. In the present study, we explored expression and possible functions of CXCL11 and its receptor CXCR3 in the human endometrium.

Method of study: First, effect of IFN- γ on CXCL11 expression in separately cultured endometrial stromal cells (ESC) and epithelial cells (EEC) were studied. CXCR3 expression in EEC, ESC and trophoblast cells was then determined. Effect of culture media of IFN- γ -stimulated EEC on migration of trophoblast cells and T cells was studied. Lastly, effect of CXCL11 on BrdU incorporation in ESC and EEC was studied.

Results: Secreted CXCL11 protein was not detected in cultured endometrial stromal cells (ESC) but was detected in cultured endometrial epithelial cells

(EEC). IFN- γ stimulated the protein levels of CXCL11 in a dose dependent manner in EEC. CXCL11 secreted from EEC with 100 ng/ml IFN- γ was 220-fold of the control. CXCR3 was expressed in EEC, ESC and trophoblast cells. Addition of IFN- γ to EEC increased the chemotactic activity of its culture media to trophoblast cells and T cells, and the effect was suppressed by immunoneutralization with antibodies of three CXCR3 ligands, including anti-CXCL11 antibody. CXCL11 significantly increased BrdU incorporation, whereas CXCL11 significantly decreased BrdU incorporation.

Conclusions: These findings suggest that IFN- γ promotes implantation by stimulating EEC to produce CXCL11, which induces migration of trophoblast cells and T cells, proliferation of ESC, and apoptosis of EEC.

ICRI-07-2-78

Survivin expression during murine pregnancy

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Problem: Survivin belongs to the family of inhibitor of apoptosis protein (IAP) and its expression is up-regulated during proliferation. Its augmented expression, moreover, correlates with a poor outcome in a variety of human tumors. We have recently shown that active vaccination against survivin protects against neuroblastom in a syngeneic immunocompetent murine model. Due to the similarities in proliferating ability between tumors and the growing placenta we hypothesized that survivin may play a role in allowing fetal and placental growth as well as in the survival of the semiallograft tissue within the uterine tissue.

Method of the study: To prove principle of concept, we first analyzed the mRNA and protein levels of survivin in uterine and placental tissue before and during murine pregnancy. Second, we compared tissues from normal pregnant (CBA/J x BALB/c) and abortion-prone animals (CBA/J x DBA/2J) at different time points.

Results: We observed a ~ 20 fold increase in mRNA expression of survivin on day 5 of pregnancy

compared to tissues of non-pregnant animals. Protein levels were increased in placental tissue on day 8 of pregnancy. In later pregnancy stages (days 10, 14 and 18) survivin levels decreased to levels comparable to those observed before pregnancy. No statistical significant differences could be determined between normal pregnant and abortion-prone animals at any time point.

Conclusions: Our data suggest an important role of survivin in proliferation of the implanting trophoblasts. Future functional studies using primary trophoblasts will shed more light onto the function of survivin during implantation and pregnancy.

ICRI-07-2-79

Tumor associated glycoprotein-72 inhibits the maturation and induces anti-inflammatory properties of decidual CD1a+ dendritic cells

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Problem: The aim of this study was to evaluate the effect of physiologically present glandular mucin, tumor associated glycoprotein-72 (TAG-72), on the phenotype and function of decidual CD1a+ dendritic cells (DC).

Methods of study: Distribution of CD1a+ cells was analyzed by immunohistology in normal human first trimester pregnancy decidua. Decidual mononuclear cells (DMC) were obtained by enzymatic digestion and gradient density centrifugation. The phenotype and cytokine production in decidual CD1a+ DC was analysed by flow cytometry in DMC cultured in the presence of TAG-72 (200U/mL) or in the medium only for 18 hours. Decidual CD1a+ cells magnetically separated (purity ~40%) from the adherent DMC fraction were untreated or TAG-72 treated, and co-cultured with CD6 purified cord blood or decidual T cells for 3 or 6 days. Polarization of T cells was measured by analysing IL-4, IFN- γ and CD45RA expression.

Results: Decidual CD1a+ cells within decidua are found in a close contact with glandular cells. TAG-

72 decreased the expression of maturation markers (CD83, CD80, CD86 and HLA-DR) on CD1a+ DC. The production of IL-15 and IFN- γ was decreased, IL-10 was enhanced, whereas IL-18 was unaffected in CD1a+ DC pretreated with TAG-72. TAG-72 pretreated CD1a+ DC increased IL-4 and abolished IFN- γ production in cord blood and decidual T cells after 3 and/or 6 days of culture at the DC/T cell ratio 1:5. Concomitant decrease of CD45RA expression on the surface of CD3+ cells was noticed.

Conclusions: TAG-72 inhibits the maturation and induce anti-inflammatory properties of decidual CD1a+ DC. The experiments are partially financed by Croatian Ministry of Science (Grants No. 0620402-0376 and 0377) and EMBIC project, European FP6, NoE, No. 512040, LSHM-CT-2004-512040.

ICRI-07-2-80

Enhancing effects of syngeneic pregnancy and peptidoglycan-monomer linked with zinc on regulatory T cells in the maternal liver

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Problem: Regulatory T cells (Treg) were claimed to be important players in the tolerance towards the fetus bearing alloantigens, having the pivotal role in inhibiting inappropriate activation of cell- and antibodies-mediated immune responses against self-antigens, as well as innate immune responses. They, however, might be related also with fetal invasion mechanisms at the maternal-fetal interface, as well as with the systemic hormonal changes, induced by pregnancy, showing that they reflect more global changes of the maternal immune system.

Material and methods: In an attempt to enlarge our previous findings showing that during syngeneic pregnancy in the liver accumulate cytotoxic self-reactive NKT cells (Mrakovcic-Sutic et al. Scand J Immunol. 2003;58:358-66;), which usually cooperate with Treg cells, in this study we estimated the presence of CD4⁺CD25⁺Foxp3⁺ T cells in the liver, spleen and thymus of nonpregnant and pregnant C57/BL6 females mated with C57 BL/6 males.

Phenotypic analysis and cytotoxicity against syngeneic thymocytes and YAC-1 targets was made on the 16th day after detection of vaginal plugs.

Results: The data have shown that during syngeneic pregnancy in the maternal liver increases the proportion of CD4⁺CD25⁺Foxp3⁺, as well as CD3^{intermediate}, NK1.1⁺ and IL-2Rβ⁺ cells (NKT cells). Moreover, the treatment of mice with PGM-Zn additionally increased the Treg accumulation particularly in the liver. Furthermore, these findings correlated with the expression of heat shock protein gp96 in the liver and in the spleen.

Conclusion: The data provide the new evidence about the interaction of NKT cells, that recognise self glycolipid antigens and immunosuppressive Treg cells and point to the liver as a place important for the activation of tolerance-promoting mechanisms during the pregnancy. Supported by Croatian Ministry of Science, Grant No. 0621341-1337.

ICRI-07-2-81

Macrophage regulation of embryo adhesion molecule expression in human endometrial epithelial cells

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Problem: The uterine endometrium undergoes dramatic structural and functional changes to allow the embryo to attach and implant in early pregnancy. The molecular processes occurring at implantation are critical in controlling early trophoblast invasion and ensuring adequate placental development. We hypothesise that macrophage populations resident in the endometrial tissue influence local cell communication and tissue remodeling events to promote uterine receptivity for embryo implantation. In this study we have investigated the role of macrophages and their secreted products in the regulation of epithelial cell expression of adhesion and anti-adhesion molecules.

Method of study: Endometrial epithelial RL95-2, HEC1A and Ishikawa cell lines were co-cultured alone or in the presence of LPS- or PMA-activated or resting human monocytes separated spatially using transwell inserts. Expression of several genes encoding adhesion and anti-adhesion molecules, glycosylation enzymes, and steroid hormone receptors were

measured by quantitative real-time RT-PCR using SYBR green chemistry.

Results: Integrin beta 1, beta 3 and estrogen receptor beta mRNA expression were increased by PMA-stimulated monocytes. Fucosyl transferase (FUT) 1 and FUT2 mRNA levels were increased by co-culture with LPS-stimulated monocytes and resting monocytes. The three cell lines responded in a qualitatively similar manner to culture with the different macrophage preparations.

Conclusion: These results suggested that macrophage-derived cytokines or other signaling factors might facilitate development of an implantation-receptive endometrium via regulation of embryo adhesion molecule synthesis in epithelial cells. Dysregulation in the phenotypes or abundance of uterine macrophages might provide an explanation for some forms of primary unexplained infertility.

ICRI-07-2-82

Phenotypic and functional characteristic of the first trimester pregnancy decidual CD1a+ dendritic cells

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Problem: The study was aimed to analyse phenotype and functional properties of the first trimester normal human pregnancy decidual CD1a+ dendritic cells (DC).

Method of study: Decidual mononuclear cells (DMC) are obtained by enzymatic digestion and gradient density centrifugation. The phenotype of CD1a+ DC (differentiation and maturation markers, as well as NK cytotoxicity receptors) was analysed immediately after the isolation and in both: adherent and non-adherent DMC fraction obtained after the 18-hour culture *in vitro*. In freshly isolated DMC, cell permeabilisation method with simultaneous surface CD1a labeling was used for cytokine and cytolytic mediator detection.

Results: Decidual CD1a+ cells are lin-1 negative and show mostly myeloid, immature DC phenotype according relatively low CD83, CD80, CD86, HLA-

DR and high CD14 molecule expression. There is no significant difference in the phenotype of CD1a+ DC between adherent and nonadherent fraction. CD1a+ cells from freshly isolated suspensions express mostly IL-15 and IL-18 cytokines, the percentage of IFN- γ + and TNF- α + cells was approximately 2 times lower and the percentage of IL-10 producing cells was negligible. CD1a+ cells are equipped with short and long term cytolytic mediators, as well as surface NK cytotoxicity receptors.

Conclusion: Mostly immature CD1a+ DC represent a source of pro-inflammatory cytokines and could be involved in cytotoxicity reactions in decidua according to their equipment with cytolytic armentarium. The experiments are partially financed by Croatian Ministry of Science Grant No. 0620402-0376 and EMBIC project, European FP6, NoE, No. 512040, LSHM-CT-2004-512040.

ICRI-07-2-83

Macrophage regulation of epithelial cell fucosyltransferase expression in mouse endometrium during early pregnancy

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Problem: The embryo must attach to and then breach the barrier posed by the epithelium of the maternal reproductive tract to establish connections vital for further development. In mice, exposure to seminal fluid mediates the recruitment and activation of macrophages into the stromal tissue sub-adjacent to the luminal epithelium. When recruitment and activation of macrophages in early pregnancy is inhibited by ablation of the seminal stimulus, implantation is compromised, placental development is altered, and fetal and post-natal growth are adversely affected. This project seeks to investigate the precise role of macrophages in the endometrial tissue remodelling processes underpinning optimal embryo implantation.

Method of study: Expression of mRNAs encoding various adhesion and anti-adhesion molecules implicated in implantation success, including integrins α v, α 4, β 1 and β 3, fucosyltransferases (FUT)-1, FUT2,

and FUT4 and mucins (MUC)-1 and MUC4 from uterine epithelial cells isolated from whole uteri, cultured alone or with macrophages or macrophage conditioned media for 24 and 48 hours were quantitated. RNA was processed for real-time quantitative RT-PCR, and data normalised to β -actin. In addition RNA from luminal epithelium laser-microdissected from uteri on day 4 post-coitum with intact males or seminal vesicle deficient males was analysed by real-time RT-PCR.

Results: Macrophage co-culture significantly increased epithelial FUT2 and FUT4 mRNA expression at 48 hours and the effect of co-culture was replicated with macrophage conditioned media. Mating with seminal vesicle deficient males resulted in a 4-fold reduction in epithelial FUT2 mRNA expression, as well as a 2-fold reduction in MUC1 mRNA expression on day 4 post-coitum.

Conclusions: These data demonstrate that exposure to seminal fluid may regulate endometrial epithelial phenotype in preparation for embryo implantation and that the effect is mediated via the agency of factors secreted from macrophages recruited during the inflammatory response to insemination.

ICRI-07-2-84

Enhanced expression of metallothioneins I/II in placenta and maternal liver, induced by syngeneic pregnancy and by peptidoglycan monomer linked with Zn in mice

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Problem: Metallothioneins (MTs) are a class of small, cysteine-rich proteins that have been implicated as a transient response to any form of stress or injury providing cytoprotective action against oxidative injury, DNA damage and apoptosis. Moreover, through the sequestration and the release of Zn and other metals MTs play an important role in the attenuation or amplification of various transcription factors, metalloenzymes, and growth factors that are activated during mammalian development and are required for repair and rapid cell growth. Furthermore, they participate in immune homeostasis,

affecting the functions of several immune cells, TLR signaling, upregulation MAC proteins and co-stimulatory molecules on dendritic cells and the production and release of pro-inflammatory cytokines.

Material and methods: Owing to the possibility that MTs contribute to the structural integrity of the placenta and participate in regulation of maternal immune response, in this study, using the immunohistochemistry, we analyzed the tissue distribution of MTs I/II on 16th day of syngeneic pregnancy (placenta, liver, thymus and spleen). Additionally, to analyze the possible influence of Gram (+) bacteria on these events, on gestation days 10, 12 and 14 mice were treated with PGM-Zn.

Results: The data have shown that syngeneic pregnancy induces high cytoplasmic and nuclear expression of MTs I/II in maternal liver, as well as in fetoplacental unit, affecting syncytiotrophoblast, cytotrophoblast, stromal and macrophages-like cells within the core of the chorionic villi and some decidual cells. PGM-Zn highly upregulated the findings in placenta, as well as in maternal liver and increased the number of MT-expressing cells in the thymus.

Conclusion: The data underline the link between the maternal liver and fetoplacental unit and suggest that MTs might be involved in the processes of morphological differentiation within the villous placenta, protection against gestational infections, and/or other damage-associated signals. (Supported by grant 0621341-1337 from Croatian Ministry of Science).

ICRI-07-2-85

A three dimensional invitro model system as a predictive assay for pregnancy-related complications

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Objective: Pre-eclampsia (PE) is a major cause of maternal and fetal mortality, morbidity, and its clinical manifestations are hypertension, proteinurea, and kidney pathology. Vascular dysfunction, shallow decidual invasion of trophoblasts, and poor spiral artery transformation are considered to play an important

role in programming the patho-physiology of PE. The objective of this study was to develop a simple *in vitro* system involving interactions between endothelial cells, invasive trophoblasts and possibly decidual NK cells that can be used as a predictive assay for PE.

Method: First trimester human trophoblast HTR-8 cells and human umbilical vein endothelial cells labelled with cell trackers, were co-cultured on matrigel for 12 hr with 10% term normal pregnancy serum (NPS), serum from mild and severe PE patients, or anti-angiogenic factors. Spontaneous tube formation was monitored by immunofluorescence microscopy and quantified.

Results: Spontaneous remodelling exhibited by trophoblasts takes place only in the presence of endothelial cells and follows the latter's footprint in action. The capillary tube formation could be disrupted by PE serum or anti-angiogenic factors and rescued by IL-10 or treatment of serum by heparin. A pilot longitudinal study indicates that serum samples obtained at 7 or 14wk of gestation from patients who were eventually diagnosed with PE can disrupt tube formation, documenting the significance of the assay system. Cells pre-treated with PE serum showed a significant inhibition of VEGFR2 phosphorylation.

Conclusions: Thus, our three dimensional culture system offers a sensitive method to assay anti-angiogenic properties in PE serum, even at very early stages of pregnancy.

ICRI-07-2-86

Altered expression of metallothioneins I/II and HLA-DR in uterine cervical squamous lesions

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Problem: Cervical intraepithelial neoplasia (CIN) has been described as premalignant lesion, from which may develop cervical cancer, the second most common cancer among women. In many cases, mildly and moderately dysplastic areas (CIN I and CIN II) regress spontaneously, but CIN III is regarded

as being identical to carcinoma in situ (CIS). Owing to the findings that various human tumours show an increased expression of metallothioneins (MTs) and MHC class II antigens, which might be associated with protection against DNA damage, oxidative stress and apoptosis, or with the activation of an immune response, respectively, in this study the immunohistochemical expression of MT I/II and HLA-DR was investigated in CIN grades I-III, and in invasive cervical squamous carcinomas.

Material and methods: HLA-DR and MTs expression was analyzed by immunohistochemistry on formaldehyde-fixed uterine specimens comprising 5 women with mild dysplasia (CIN I), 5 women with moderate dysplasia (CIN II), 10 women with severe dysplasia (CIN III), and 9 women with cervical carcinoma. The intensity of staining was graded on an arbitrary scale from negative (0) to strongly positive (5+).

Results: Aberrant expression of HLA-DR and cytoplasmic MTs I/II expression were found on cells in lamina propria and in endocervical glandular epithelium in intensity, which increased starting from CIN I toward CIN III. In CIS, however, the HLA-DR expression of high intensity was less frequent, and nuclear staining of MTs was noticed.

Conclusions: The results suggest that increased expression of HLA-DR and MTs may be related to the evolution of cervical cancer, as well as that MT might have regulatory effects on MHC-class 2 expression in these events. (Supported by grant 0621341-1337 from Croatian Ministry of Science).

ICRI-07-2-87

The role of lectin-complement pathway in innate immune responses in clinical preeclampsia

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Problem: In preeclampsia, factors/mediators are released from poorly perfused placenta into maternal circulation, excessive inflammatory and oxidative-

stress responses and generalized endothelial dysfunction are resulted. However the circulating factors have yet to be confirmed and origin of the inflammatory responses is unclear. Our aim was to identify the circulating factors from preeclamptic women, and investigate their relationship to underlying immune response for the development of preeclampsia.

Method of study: We undertook comparative proteomic analyses to display abnormal circulating factors from preeclamptic women by 2D-PAGE and MALDI-TOF-MS/MS. We evaluated the potential role of the candidate proteins identified in preeclamptic placenta by western and immunohistochemical analyses. We investigated their immune responses using pro-inflammatory cytokine antibody arrays.

Results: We found that ficolins, the pattern recognition proteins involved in the lectin-complement pathway, were differentially expressed in plasma from preeclamptic pregnancies. Ficolins were maintained at very low concentrations in plasma, but were found at high levels in the placenta, particularly in syncytiotrophoblasts undergoing apoptosis. The specific binding of ficolins to MASP receptors, rather than cC1q, in apoptotic trophoblasts anticipated in the clearance of the cells through lectin-complement activation and induced innate immunity through local and systemic innate pro-inflammatory cytokines and chemokines activation. The changes were significantly associated with the clinical manifestation of preeclampsia.

Conclusions: We identified the abnormal pattern recognition lectins in preeclamptic women. Lectins were involved in specific-binding and clearance of apoptotic trophoblasts in preeclamptic placenta which were responsible for the local immune recognition and systemic inflammatory response in the development of clinical preeclampsia.

ICRI-07-2-88

Dendritic cells, Interleukin-12 and soluble CD40 Ligand in pre-eclampsia

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Problem: It has been shown that pre-eclampsia is Th1 type immunity disorder. Recently two distinct

lineages of dendritic cells (DCs) have been described in humans. Myeloid DCs can drive T cell differentiation into Th1 lymphocytes. Lymphoid DCs can induce Th2 immunity. The aim of the study was to estimate myeloid and lymphoid DCs and serum concentrations of IL-12 and soluble CD40 Ligand in peripheral blood of patients with pre-eclampsia and healthy third trimester pregnant women.

Method of the study: Twenty five pregnant patients with pre-eclampsia and twenty healthy pregnant women were included in the study. Dendritic cells were isolated from peripheral blood, stained with monoclonal antibodies against blood dendritic cell antigens (anti BDCA-1, BDCA-2, BDCA-4) and estimated using the flow cytometric method. The concentrations of Interleukin-12 and soluble CD40 Ligand were estimated using immunoenzymatic ELISA method.

Results: Myeloid BDCA-1⁺ and lymphoid BDCA-2⁺ DCs were present in peripheral blood of patients with pre-eclampsia and healthy third trimester pregnant women. The percentage of lymphoid dendritic cells was significantly lower in pre-eclampsia in comparison with control group, while BDCA-1⁺:BDCA-2⁺ cell ratio was significantly higher in pre-eclamptic patients when compared to control groups. The concentrations of IL-12 and CD40 Ligand were significantly higher in sera of patients with pre-eclampsia when compared to control group.

Conclusions: It seems possible that lower BDCA-2⁺ cells percentage, higher BDCA-1⁺:BDCA-2⁺ cell ratio and elevated concentrations of IL-12 can be associated with increased Th1 type immunity in patients with pre-eclampsia. Elevated concentrations of CD40 Ligand can be responsible for altered function of dendritic cells in pre-eclampsia.

ICRI-07-2-89

Comparison of peripheral blood and endometrial NK cells specific markers' expression between Recurrent Spontaneous Abortion and Primary Infertility Women

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Problem: Cytotoxicity mediated by Natural Killer (NK) cells has been implicated in the unsuccessful reproduction pathophysiology, being the decidual NK cells (CD16⁻CD56^{bright}) the main subject related to the immunomodulatory effect at the fetal-maternal interface. The objective of this study was to compare the peripheral blood (PB) and endometrial NK cells markers' expression between Recurrent Spontaneous Abortion (RSA) patients and women with Primary Infertility (PI).

Methods: For purpose of this study, RSA (n=10) was considered as ≥ 2 losses during the first 20 weeks of pregnancy, and PI (n=22) at least 2 unsuccessful in vitro fertilization procedures. NK cells immunophenotyping was performed by flow cytometry (BD-FACSCalibur) simultaneously in PB and secretory endometrial specimen, evaluating among total lymphocytes, the percentage of CD45⁺CD3⁻ cells expressing the following markers: CD16⁺, CD16⁺CD56⁺, CD16⁻CD56^{bright} and CD16⁻CD56^{dim}, respectively. Non-parametric tests were applied for comparisons (Wilcoxon and Mann-Whitney) and correlation (Spearman), being significant P values ≤ 0.05 .

Results: The relative number of each NK cells' phenotype was significantly different between PB and endometrial (P=0,000). There was no correlation between both cell sources (P $\geq 0,172$), except for the CD16⁺CD56⁺ phenotype (R=0,484; P=0,005). The same analysis did not show any difference comparing RSA and PI patients.

Conclusions: Endometrial NK cells (number/phenotype) can not be predicted from its PB analysis, and PI patients had the same spectrum of endometrial NK cells as RSA patients. These data suggest that if

there is some benefit from immunomodulatory therapies for RSA based on the cytotoxicity immunomodulation, PI patients could also be suitable for specific studies.

ICRI-07-2-90

Is human chorionic gonadotropin (hCG) truly a tumor-associated antigen in non-germ cell tumors?

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Circulating hCG is associated with several types of cancer, including placental and germ-cell trophoblastic tumors in which the origin of the malignant cells provides a ready explanation for hCG expression. However, the serum of 10-60% of patients with a variety of non-trophoblastic cancers contain either hCG $\alpha\beta$ heterodimer or, more commonly, hCG β alone (Stenman *et al.* Clin Biochem. 2004;37:549-61). This material could either be produced by the tumor or by non-tumor cells as a response to factors secreted by the tumor. We carried out a survey of genome-wide searches for mRNA expression in tumors. Surprisingly, such approaches have so far failed to detect substantial transcription of hCG β -chain genes in most non-trophoblastic (including colorectal and bladder) cancers, even though specifically-targeted amplification of hCG β 3/9, 5 and 8 gene transcripts have led to their detection in bladder cancer (Hotakainen *et al.* Tumor Biol. 2007;28:52-56). We believe that the failure to find hCG β gene expression in genome-wide searches of tumors is explained by the fact that only a small proportion of the tumor cells aberrantly express hCG β . Immunohistochemical staining of primary tumor tissue from urogenital cancers indicates that hCG β producing cells are situated at the invading edge of the tumor mass and more closely associated with neo-vascularisation. We propose that the hCG β -expressing cells represent an invasive phenotype, thereby explaining the efficacy of hCG vaccination in prolonging survival in patients with metastatic colorectal cancer (Moulton *et al.* Clin Cancer Res. 2002;8:2044-51).

ICRI-07-2-91

Analysis of the relationship between ureaplasma infection and premature birth

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Problem: *Ureaplasma urealyticum* has been recognized as a pathogen for chorioamnionitis and premature birth. In this study, we studied the mechanism of premature birth by ureaplasma infection.

Method of study: 330 pregnant women (including 300 normal pregnant women and 30 women with premature birth) were recruited for the study of the relationship between ureaplasma infection and premature birth. Vaginal swabs were obtained from 330 pregnant women after acquiring the informed consent, and the analysis of ureaplasma infection using ureaplasma culture medium (PPLO medium) was performed. *Ureaplasma* (sero type 3) was kindly supplied from the National Institutes of Health in Japan. After incubation, Ureaplasma Lipoprotein (LP) was prepared using Triton X-114 phase separation method. The monocyte cell line (THP-1) were incubated with LP for 24 hours, and then mRNA was isolated. The expression of Toll like receptor (TLR)-2 and 4 were analyzed using the real time PCR. The production of IL-8 in THP-1 was also studied by ELISA.

Results: Ureaplasma infection was identified in 90/300 (30.0%) of normal pregnant women and 15/30 (50%) of pregnant women with premature birth respectively. The infection rate in both groups was significantly different ($p < 0.05$). Ureaplasma LP significantly induced the expression of TLR-2 on THP-1 cells, but not TLR-4. Ureaplasma LP also significantly induced the production of IL-8 in THP-1 cells.

Conclusion: In this study, we confirmed that the ureaplasma infection caused the premature birth and demonstrated that ureaplasma LP induced the TLR-2 and cytokine IL-8 in THP-1 cells. In conclusion, Ureaplasma infection seems to be an important factor in premature birth by inducing the pro-inflammatory cytokines.

ICRI-07-2-92

Igf2 and H19 gene polymorphisms in couples with recurrent spontaneous abortion

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Problem: Genetic markers could be factors of predisposition to idiopathic recurrent spontaneous abortion (RSA). Igf2 and H19 genes undergo the phenomenon of *genomic imprinting*, Igf2 being expressed from paternal, and H19 only from maternal allele. Igf2 is a major fetal growth factor that stimulates angiogenesis, proliferation and transport of nutrients in placenta. H19 is a non coding gene whose untranslated RNA suppresses growth by controlling the level of Igf2 gene expression and Igf2 mRNA cytoplasmic localization. The aim of this study is to evaluate the association between Igf2 and H19 gene polymorphisms and the susceptibility to RSA.

Method of study: A case-control study was conducted to determine the association between Igf2 and H19 gene polymorphisms and the risk of RSA in 113 couples with RSA, and 113 fertile couples as control. PCR was performed to analyse DNA for Igf2 ApaI polymorphism in exon 9 and H19 HhaI polymorphism in the 6th CTCF binding site.

Results: Statistically significant difference was found for Igf2 ApaI polymorphism in male partners of RSA women. Their genotype frequencies compared with controls were: 13%/56%(AA); 39%/20%(GG); 48%/25%(AG); when following dominant model for risk genotypes (AA+AG), OR=0.38, p=0.001.

Conclusion: Considering that Igf2 gene is expressed only from paternal allele, the ApaI polymorphism in partners of RSA women could affect Igf2 level of expression in placenta and lead to RSA. The research of polymorphisms in genes undergoing the phenomenon of genomic imprinting and including male partners, represents a new approach in the research of genetic etiology of idiopathic RSA.

ICRI-07-2-93

Counting T lymphocytes, B lymphocytes and Natural Killer cells in peripheral blood and endometrium in Recurrent Spontaneous Abortion and infertile patients

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Problem: Natural Killer cells (NK: CD16+CD56), T and B lymphocytes were quantified among the peripheral blood and endometrial lymphoid cells, comparing Recurrent Spontaneous Abortion (RSA) and women with Primary Infertility (PI) and correlating their distribution between both cell sources.

Methods: Among 273 patients, 119 had RSA and 154 were PI.

Results: Blood cells distribution was similar between RSA and PI. PI had a higher amount of endometrial NK cells compared to RSA patients (P=0,018). The prevalence of associated thrombophilia was the same between both groups, and it was not associated with a different cell distribution pattern neither in blood nor at the endometrium. Eventhough B lymphocytes among RSA patients and NK cells among PI achieved a statistically significant Spearman correlation between blood and endometrium (P=0,037 and P=0,045, respectively), they did not achieve any clinical impact, being R=0,193 and R=0,162, respectively.

Conclusions: We conclude that Pi have a higher amount of endometrial NK cells, and that the endometrial lymphoid cells distribution cannot be predicted by the peripheral blood analysis.

ICRI-07-2-94

Autoimmune Thyroiditis Developing in a Survivor of Catastrophic Antiphospholipid Syndrome: A Case Report

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Rationale: We report a case of an autoimmune thyroiditis developing in a patient who survived an episode of Catastrophic Antiphospholipid Syndrome (CAPS), which is a life-threatening, multi-organ thrombosis occurring over a short period. A Medline search failed to show prior reported events. The search, however, did reveal a few reports of an association between the Antiphospholipid Syndrome (APS) and thyroiditis.

Method: A Case Report

Results: This is a case of a 31 year-old female, G2P1 (1001), who had a history of hyperthyroidism 2 years prior and was treated with methimazole for 4 months, rendering her euthyroid. FT3 was found to be low on her 9th week of this pregnancy, but it normalized during her 24th week without any medications. Post-caesarean delivery, she developed sudden hypotension, oliguria, dyspnea, tachypnea and bradycardia. Laboratory findings showed thrombocytopenia, elevated liver enzymes, prolonged activated partial thromboplastin time (aPTT), elevated D-dimer and Troponin-I, and a multiseptal wall motion abnormality and an ejection fraction of 30% on echocardiography. A diagnosis of Catastrophic Antiphospholipid Syndrome was made, and she was treated with enoxaparin, hydrocortisone, and intravenous immunoglobulin (IVIG) with dramatic improvement of symptoms and return to normal of the laboratory findings. Eight months later, she developed hypo/hyperthyroidism and high titers of anti-thyroid antibodies, confirming the diagnosis of an autoimmune thyroiditis. She was treated with thyroid hormones and eventually underwent a total thyroidectomy.

Conclusion: This case report identifies an association between 2 autoimmune phenomena, namely, the catastrophic antiphospholipid syndrome and autoimmune thyroiditis.

ICRI-07-2-95

Frequency of autoantibodies in unsuccessful *in vitro* fertilisation

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Problem: One of the solutions of the problem of infertile couple is the *in vitro* fertilization (IVF) followed by embryo transfer (ET). The success of intervention, i.e. the existence of pregnancy, requires the optimized coexistence of several endocrine, genetic and technical factors. In most cases of unsuccessful IVF-ET, immunological deviations, such as latent autoimmune diseases or circulating antibodies that prohibit the embryo implantation, may be considered in the background.

Method of study: The sera of 100 women with 3 or more unsuccessful IVF-ET intervention were analyzed by ELISA or IFA for the following antibodies: ANA (anti-nuclear antibody), ds-DNA, coeliac specific antibodies (EMA, tTG), thyroid antibodies (TPO, HTG), cardiolipin, β 2GPI and anti-laminin-1.

Results: 35% of examined women proved to have positive thyroid gland antibody levels with normal TSH levels. Positive phospholipid antibody, and laminin antibody values were found in 3% and 2% of the cases respectively, ANA tests proved to be positive in 2% of the cases. No positive coeliac specific antibody was found in the examined group.

Conclusions: The treatment of women proved to have positive TPO and/or HTG antibodies resulted in 9 cases (25.7%) ongoing pregnancy. We suggest screening to thyroid gland antibodies before IVF-ET, because of the high rate of positive tests and high rate of pregnancy due to the treatment based on the immunological check-up.

ICRI-07-2-96

Are alloantibodies harmless beside the induction of operative tolerance by partner specific platelet transfusion in RSA with proved alloreactive background?

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Problem: Major part of Recurrent Spontaneous Abortion (RSA) cases are possessing immunopathogenic background. The missing protective immune regulation could be achieved by immunotherapy either with Intravenous Immunoglobulin (IVIG) or partner specific transfusion. The question arises whether HLA or other tissue specific alloantibody production as side effect in consequence of transfusion is disadvantageous or not.

Method of study: RSA cases with proved alloreactivity background were transfused with partner specific purified platelet suspension. Before and after transfusion FcR II blocking (EAI) antibodies, moreover HLA- and platelet allospecific antibodies were detected.

Results: All RSA cases treated with three or less partner specific platelet product produced EAI antibodies. Half of the cases in the presence of EAI antibodies produced HLA or/and platelet specific (HPI) alloantibodies, as well. Since it is not clarified whether these alloantibodies are influencing the induction of operative tolerance or are harmful to the patient or to the fetus they were turned to IVIG treatment. In general, partner specific platelet transfusion alone was efficient to protect pregnancy. In the cases with alloimmunization the additional IVIG treatment was efficient and the alloantibody disappeared, moreover dose reduction was possible.

Conclusions: In RSA cases with proved alloreactive background the partner specific platelet transfusion could be an efficient and proper immunotherapy to induce the protective immunoregulation for the fetus. However, in certain cases it induces partner specific alloantibodies as a side effect. The question remains open whether those alloantibodies are harmless or harmful and whether they need additional suppressive immunotherapy or not.

ICRI-07-2-97

Defining alloimmune and autoimmune background in recurrent pregnancy loss leads to successful intravenous immunoglobulin immunotherapy

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Problem: Immunotherapies (leukocyte immunization, intravenous immunoglobulin (IVIG)) introduced to treat women with recurrent spontaneous abortion (RSA) are still controversial in most clinical trials. A careful selection of patients is necessary for higher efficacy.

Method of study: A complex immunological panel assay was offered to patients with reproductive failure without any other known cause. The importance of cell mediated immunological parameters is outlined here beside the immunogenetic ones. The complex immunological assay and the clinical study were performed only with patients in whom the reproductive failure was realized without any anatomical, endocrinologic, genetic abnormalities, infections or intestinal diseases.

Results: High cytotoxic T lymphocyte precursor (CTLp) frequency, cell-mediated cytotoxic activity (CML) and rather high natural killer (NK) cell activity were found in alloimmune patients. About eighty patients were investigated by immunological assays and in more than 70% of the women an alloimmune background could be defined. The efficacy of IVIG treatment was higher than 90% in this group. The monitoring of the immunotherapy showed in most of the selected cases decrease in the CTLp frequency, CML and Natural Killer cell activity parallel to the increased FcR blocking antibody percent.

Conclusions: Our novel cellular immunological assays proved to be favourable for the indication of RSA patients for IVIG therapy. The selection process led to a more effective immunotherapy with significantly higher success rate.

Acknowledgements: The work was supported by the Hungarian Ministry of Welfare (ETT 172/200,

T05 219/96) and the Hungarian Ministry of Economy (GVOP-2004-3.1.1).

ICRI-07-2-98

Rheumatoid arthritis and pregnancy after assisted reproductive technology – case report

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Problem: Compelling evidence suggests that an abnormally functioning maternal immune system often leads to poor reproductive performance. Failure of proper function of immunologic interaction during implantation has been implicated as a cause of recurrent miscarriage, late pregnancy fetal loss, IVF failure and infertility. Women with risk factors such as a positive personal or family history of autoimmune disorders such as rheumatoid arthritis, lupus erythematosus and hypothyroidism (Hashimoto's disease) need properly evaluation because immunologic problems may lead to implantation failure. The selective use of immunotherapy (heparin/aspirin, intravenous immunoglobulin G, corticosteroid therapy) has enabled to achieve successful pregnancies in-patients who had previously suffered repeated IVF failures. Disease therapy and drugs continue to be a concern in the first trimester and throughout pregnancy. Some drugs can cause birth defects, others can cause miscarriages. The greatest risk of these drugs comes later in pregnancy, when they may interfere with labor, affect amniotic fluid production or cause excessive bleeding during delivery.

Method of study: We presented case report of 27 year old woman with juvenile rheumatoid arthritis and infertility problems who gave healthy term birth after second IVF procedure in our institution.

Conclusion: There are many risk for pregnancy in rheumatoid arthritis patient. Successful pregnancy is possible after assisted reproductive technology, also. Pregnancy surveillance needs multidisciplinary approach.

ICRI-07-2-99

Macrophages and blood vessels in testicular biopsies of patients with non-obstructive azoospermia

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Problem: In the current study, we wanted to investigate and correlate the presence of testicular macrophages and changes of blood vessels in testicular biopsies of patients with non-obstructive azoospermia.

Method of study: A total number of 108 patients with azoospermia were included into the study. They were divided into two groups: controls (n= 23; normal spermatogenesis) and "mixed atrophy of seminiferous tubules" (n= 85). Testis interstitium was investigated using semi- and ultra-thin sections (TEM), immunohistochemistry (IHC) and morphometry (stereology). Semi-thin sections were stained with toluidine blue in order to visualize macrophages and blood vessels. In the IHC part of the study, the following antibodies have been applied: KiM1P1 (macrophage marker), α -smooth actin, F-VIII, fibrinogen, CD31, PCNA and Ki-67. The number of macrophages and the volume, length and curvature of arteries, veins and capillaries per testis have been established by stereology. The above-mentioned variables were correlated.

Results: In the "mixed atrophy" group an increased number of macrophages could be found when compared to controls. TEM analysis indicated that macrophages were activated. The subintimal layer in some arteries and veins was significantly thickened. IHC results indicated a significant proliferative activity in the fibrotic interstitial areas of the testis with a formation of new capillaries. Statistical analysis showed that the increased presence of macrophages was significantly associated to the change in the structure of blood vessels ("mixed atrophy" cases).

Conclusion: It seems there is a close relation between testicular macrophages and the extent of pathological changes in the structure of blood vessels.

ICRI-07-2-100

Uropathogenic *E.coli* but not commensale *E.coli* infection activates TLR-4 MyD independent signaling pathways in rat testicular cells

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Problem: Immunological infertility accounts for at least 12-13% of male infertility. *Uropathogenic Escherichia coli* (UPEC) is the most frequent pathogen causing bacterial genital tract infections in men, however, the testicular defence to bacterial infection is poorly investigated.

Method of study and Results: The mRNA expression pattern of Toll-like receptors (TLRs) 2–10 was investigated in isolated testicular cells and peritoneal macrophages (PM) as TLRs function as sensors for conserved pathogen-related molecular patterns (PRMPs) such as LPS. Using RT-PCR all testicular cell types as well as PM expressed mRNAs for at least two TLRs. TLR4 protein, recognizing LPS as PRMP, was induced 2h and 6h after UPEC infection in TM and Sertoli cells (SC) or peritubular cells (PTC), respectively, whereas after infection with non-pathogenic commensale *E.coli* (NPEC) no TLR4 was detectable. When tracing the TLR signaling pathways activation of either p38, JNK and/or ERK1/2 was observed in all three cell types and PM, but only PTC and PM reacted with degradation of I κ B α . Infection with UPEC induced expression of MCP-1, IP-10 and IFN α / β in all cell types except TM indicating induction of MyD88 independent signaling pathways in SC and PTC. Surprisingly, the proinflammatory cytokines IL-1, IL-6 and TNF α were induced in PM only by LPS and NPEC, but not by UPEC. TNF α induction by LPS or NPEC could be suppressed by co-infection with UPEC.

Conclusion: We conclude from our results that UPEC but not NPEC infection is sensed by TLR4 in testicular cells. Moreover, UPEC infection seems to activate MyD88-independent signaling pathways.

ICRI-07-2-101

Relation of increased peripheral blood NK cells at the time of abortion with placental histological lesions

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Increased PB NK cell numbers have been associated to immune-mediated abortion and they are used as markers for the selection of women for immunotherapy. The aim of the study was to investigate whether the measurement of NK cells is useful when performed at the time of abortion and if their increase reflects the immunopathology at the foeto-maternal unit. Blood samples were collected from 113 women at day 5 after the vacuum curettage they had for therapeutic termination of 1st trimester missed pregnancy (Group A=79) or for elective termination of normal pregnancy (Group C=34). The samples were used to determine the percentage of CD3-CD16+CD56+ NK cells. Additionally, the abortion material was examined for the detection of lesions indicating immune-associated abortion (villitis, thromboses, necroses) scored 0–3. The results revealed that: a) More women in group A had an increased percentage of NK cells (>12%) and pathology score ≥ 2 than those in group C ($p=0,038$ and $p=0,000$ respectively). b) The measurement of the NK percentage in relation to the placental histological lesions compatible with immune reactions (test used as gold standard) was found to have a rather insignificant sensitivity and specificity (70% and 73,68% respectively), but a relatively good positive prognostic value (89,39%) as well as negative prognostic value (43,75%). An increased NK cell percentage 5 days after the termination of the pregnancy could be used as a marker of the immune aetiology of the abortion, since the probability of an aborter with NK cells >12% to have had an immune-mediated abortion is close to 90%.

ICRI-07-2-102

Polymorphisms in the Interleukin-12 and IL-18 Genes and Recurrent Spontaneous Abortion

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Problem: IL-12/IL-18 are involved in uNK control of uterine vascular development. Lower doses of IL-12/IL-18 are required for successful embryo development, while lack or high values of IL-12/IL-18 induce defects in uterine vascular remodeling during implantation and lead to pregnancy failure. Polymorphisms in the IL-12/IL-18 genes could modify the cytokine balance which might result in an increased susceptibility to recurrent spontaneous abortion (RSA).

Method of study: A case-control study was conducted to determine the association between the IL12 and IL18 (-607C>A, -137G>C) gene polymorphisms and the risk of RSA in 125 women with RSA and 136 controls. Polymerase chain reaction was performed to analyze genomic DNA for IL12B promoter insertion/deletion polymorphisms and IL18 (positions -607C>A, -137G>A) gene polymorphisms.

Results: The frequencies of CC, CA, AA genotypes for IL-18(-607) were: 34.4%, 54.4% and 11.2% in patients versus 30.1%, 58.1% and 11.8% in controls; the frequencies of GG, GC, CC genotypes for IL-18(-137) were: 47.2%, 43.2% and 9.6% respectively in patients and 45.6%, 46.3% and 8.1% in controls; the frequencies of DD, ID, II for IL-12 were, 25.6%, 52.8% and 21.6% in patients versus 21.3%, 51.5% and 27.2% in controls. When following recessive model for risk genotypes the results were as follows: for IL-18(-607 AA) OR=1.05, p=0.89; for IL-18 (-137 CC) OR=1.21, p=0.46; and for IL-12 (DD) OR=1.26, p=0.41).

Conclusion: We found no significant association between IL-12 (ins/del) and IL-18 (-607 and -137) gene promoter polymorphisms and susceptibility to RSA in studied women.

ICRI-07-2-103

Genetic polymorphisms in RSA susceptibility – a systematic review of immunity-related genes

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Problem: The genetic background of recurrent spontaneous abortion (RSA) has been widely investigated and confirmed through its familial occurrence, linkage analysis and epidemiologic studies. A polygenic inheritance has been suggested and a number of association studies have been performed with the aim of identifying candidate genes, including genes coding coagulation factors, oxidative stress, vasoactive substances, endocrine and metabolic factors and genes involved in immunological processes.

Method of study: In order to avoid contradictions between reports, uncertainties regarding false positive or false negative associations, and to ensure certainty in statistical analyses due to sample sizes, a systematic review of the studies on candidate genes related to immunology involved in RSA was performed. The PUBMED and EMBASE databases were searched up for studies on candidate genes in RSA. All case-control studies were registered and those satisfying principles of evidence based medicine were included in further analysis.

Results: Up to December 2006, 172 citations were registered; while 65 among them were studies of genetic polymorphisms in immunity-related genes. The most frequently investigated genes included HLA system (25), TNF α (8), IL-10 (7) and IL-6 (6). Large variation among reported results in studies was found.

Conclusions: The magnitude of the association between polymorphism in the immunity-related genes and RSA varies, according to the type of RSA and the type of gene/ polymorphism investigated. Further studies are needed to assess polymorphisms in immunity-related genes as the RSA risk factors.

ICRI-07-2-104

Immunotherapy of advanced stage tumours of diverse origin by a recombinant chimeric antibody against β -hCG without cross-reaction with healthy cells

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Increasing evidence is available on the ectopic expression of β -hCG by a variety of poorly differentiated tumours. Besides literature reports, amongst the 62 cases examined by us immuno-histochemically, 45 were positive for β -hCG. These included cancers of esophagus, kidney, parotid gland, stomach, breast, cervix, urinary bladder, testis, ovary, bile duct/gall bladder, intestines, and Non Hodgkin lymphoma. The positivity was high in advanced stage cancers 31/40 (77.5%) and these had poor prognosis and adverse survival. We reported previously the development of a recombinant chimeric antibody, cPiPP, (Ig G₁, kappa linked to mouse fab) of high affinity (Ka

$3 \times 10^{10} \text{ M}^{-1}$) and high specificity for hCG (*Human Reproduction*, 2002;17,2054–2061, *Current Science*, 2002; 82, 1452–1457). cPiPP was devoid of cross-reaction with kidney, liver, colon, gall bladder, and breast of healthy subjects. It does not react with hFSH, hTSH and has <5% cross reaction with hLH. This antibody binds with U-937 B cell lymphoma and MOLT-4 drugs refractory T Lymphoblastic leukemia cells with no binding to PBMC of healthy donors (*Current Science*, 2005 ; 89, 1571–1576.). Both types of cancer cells are observed to make α and β hCG intracellularly and live cells carry these ligands on their membranes enabling selective killing of tumour cells by the antibody with total lack of binding with normal healthy PBMC. Ongoing collaborative studies in patients of Acute and Chronic myeloid leukemia show that 4/11 patients had cells reactive with cPiPP as determined by FACS. An immuno-conjugate of cPiPP with curcumin killed in culture nearly 100% of tumour cells in the bleed as confirmed by tumour marker antibody CD33 before and after treatment without affecting normal healthy cells.