THE ROLE OF γ/δ T CELL RECEPTOR POSITIVE CELLS IN PREGNANCY

Ph.D. Thesis

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I. LIST OF ABBREVATIONS

β2-m	beta-2-microglobulin
BSA	bovine serum albumin
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescence-activated cell sorter
FITC	fluorescein-isothiocyanate
HLA	human leukocyte antigen
HRPO	horseradish peroxidase
HSP	heat shock protein
IFN γ	interferon γ
IL	interleukin
KIR	killer inhibitory receptor
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger RNA
MW	molecular weight
NK cell	natural killer cell
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PIBF	progesterone induced blocking factor
PR	progesterone receptor
TBC	target binding cell
TBS	tris buffered saline
TCR	T cell receptor
Th	T helper
TNF-α	tumor necrosis factor α

II. INTRODUCTION

1. THE ROLE OF THE DIFFERENT HLA CLASS I MOLECULES AT THE FETO-MATERNAL INTERFACE

The conditions that permit the human fetus to evade rejection by its mother's immune system have been the subject of intense interest for decades. The fetus itself does not come into direct contact with maternal tissue. It is the trophoblast, which forms the interface between the maternal and fetal compartments. This interface extends over almost the entire surface of the fetal membranes and the placenta.

The status of the placenta for MHC (major histocompatibility complex) antigens is of great importance because it enables the semiallogeneic fetus to survive within the immunocompetent mother. Since placental cells are devoid of HLA (human leukocyte antigen) class II antigens, interest has focused on the expression of HLA class I molecules. The *syncytiotrophoblast,* which provides the outer lining of chorionic villi, and is constantly bathed in maternal blood flowing through the intervillous spaces, does not express HLA antigens. No mRNA (messenger RNA) for HLA is detectable in syncytiotrophoblast. Underlying the syncytiotrophoblast there is an inner layer of cellular trophoblast, the *villous cytotrophoblast.* This is in contact with fetal mesenchyme, it is a mitotically active and forms a continuous layer during the early stages of placentation but becomes discontinuous as pregnancy progresses and exists only as isolated cells during the late third trimester. The villous cytotrophoblast has no contact with maternal tissues and there is no surface expression of HLA antigens. However there is HLA mRNA present, which indicates a post-transcriptional control. The lack of any HLA surface protein rules out the

possibility of maternal recognition of these trophoblast subpopulations by T cells. The *extravillous cytotrophoblast* is the subpopulation, which is external to the mature chorionic villi. These cells are in close contact with maternal cells of the decidua capsularis and as pregnancy goes on, they additionally get in contact with maternal cells of the decidua parietalis *(Fig.1.A.)*. Both extravillous cytotrophoblast and villous syncytiotrophoblast, in contact with maternal tissues, are directly exposed to potential, cellular and humoral, maternal antifetal immune effectors. Since the trophoblast is the only fetal tissue in direct contact with the maternal immune system, the lack of polymorphic class I and class II MHC antigens on trophoblast, is thought to be vital (1).

Immunohistochemical staining with different antibodies against HLA class I heavy chains or β_2-microglobulin (β_2-m) failed to reveal reactivity with villous cytotrophoblast cells. On the other hand extravillous cytotrophoblast cells do express HLA molecules (2-4) *(Fig.1.B.)*.

The HLA *class Ia* or *classical* gene family is composed of a group of genes whose products encode cell surface glycoproteins of MW (molecular weight) 40-45 kDa, associated noncovalently with the 12 kDa β_2-m light chain. These include the three highly polymorphic molecules HLA-A, -B and -C, which are expressed in most somatic tissues and which are able to present intracellular peptides to cytotoxic T cells (5).

Three additional class I genes, the less polymorphic HLA-E, -F and -G antigens (whose product is also associated with β_2-m) form the *class Ib* or *non-classical* group. These molecules show homology to classical class I molecules but generally have limited polymorphism, low cell surface expression and more restricted tissue

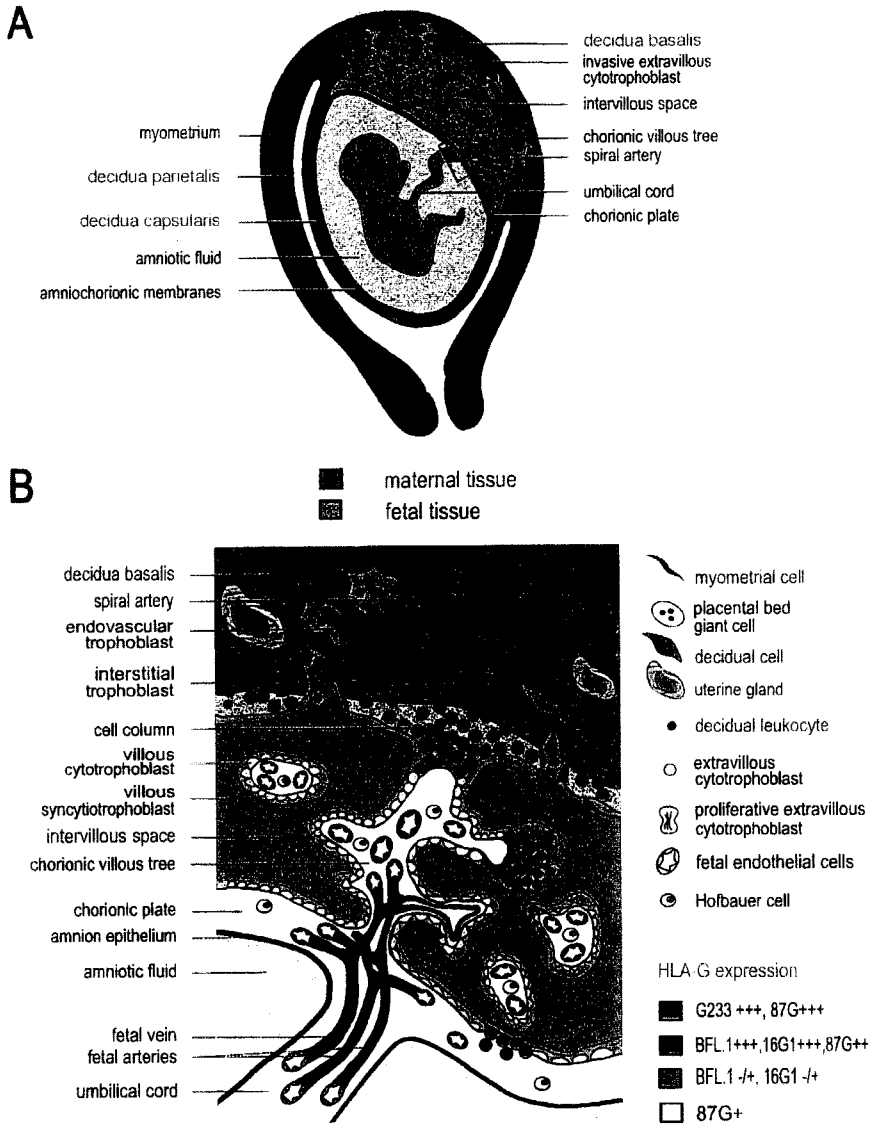
Fig.1.A. *Schematic diagram of a first trimester pregnant human uterus illustrating the different anatomical arrangements of the maternal and fetal parts of the placenta.*

The following three maternal decidual regions can be distinguished: the decidua basalis at the implantation site, the decidua capsularis overlaying the embryo and later fusing with the decidua parietalis. The fetal part of the placenta is made of chorionic villous "treelike" structures bathed in maternal blood. Most of the villi are free floating in intervillous space (floating villi); others are attached to the maternal decidua basalis (anchoring villi). Chorionic villus tree and maternal blood intervillous spaces occupy most of the placental volume. Amnion cells and the smooth chorion join together to form the amniochorionic membranes, lining the amniotic cavity in which the embryo floats during gestation.

Fig.1.B. *HLA-G expressing cells in human first trimester placenta.*

This enlarged section of Fig.2.A. (red square) shows a cross-section of an anchoring villus attached to the decidua basalis, which is composed of various maternal cell types indicated in blue. Depending on the HLA-G monoclonal antibodies used (G233, BFL.1, 87G or 16G1) different staining patterns were obtained, indicated by different colors (red, pale, green, orange or yellow, respectively).

(Le Bouteiller and Blaschitz, 1999)


A
decidua basalis
invasive extravillous cytotrophoblast
intervillous space
chorionic villous tree
spiral artery
umbilical cord
chorionic plate
myometrium
decidua parietalis
decidua capsularis
amniotic fluid
amniochorionic membranes
B
maternal tissue
fetal tissue
decidua basalis
spiral artery
endovascular trophoblast
interstitial trophoblast
cell column
villous cytotrophoblast
villous syncytiotrophoblast
intervillous space
chorionic villous tree
chorionic plate
amnion epithelium
amniotic fluid
fetal vein
fetal arteries
umbilical cord
myometrial cell
placental bed giant cell
decidual cell
uterine gland
decidual leukocyte
extravillous cytotrophoblast
proliferative extravillous cytotrophoblast
fetal endothelial cells
Hofbauer cell
HLA-G expression
G233 +++, 87G+++
BFL.1+++,16G1+++,87G++
BFL.1 -/+, 16G1 -/+
87G+

distribution (5-7). The function of these non-classical class I molecules is intensively being investigated.

Immunohistochemically HLA-G and a small amount of HLA-C were the only HLA class I molecules that could be detected in extravillous chorionic cytotrophoblast cells (8,9). Moretta and colleagues have published evidence that certain HLA-C alleles may be the protective elements on target cells against NK clones generated from peripheral blood (10).

Although the complete role and function of HLA-G and HLA-E is so far unclear, there is some evidence that the expression of both HLA-G and HLA-E have a vital importance at the fetomaternal interface.

The **HLA-G** gene exhibits the same exon-intron **structure** as seen in the classical class I genes, however the HLA-G gene has a capability to transcribe six different transcriptional isoforms, which code for four membrane bound and two soluble products: HLA-G1, HLA-G2, HLA-G3, HLA-G4 and two HLA-G_{sol} (11).

The next characteristic of HLA-G is its low **polymorphism**, only a few alleles have been described. Why is it essential? Since paternally inherited HLA-G is present on the trophoblast cells, it should be recognized as foreign by the maternal immune system. The limited HLA-G polymorphism ensures that paternal and maternal HLA-G are extremely similar or identical and hereby paternal HLA-G does not induce a maternal alloresponse. HLA-G polymorphism may be critical for protection against pathogen infection during pregnancy.

The **cellular localization** of HLA-G during pregnancy is well established. The precise localization of HLA-G proteins in human placenta was recently rendered possible by the use of specific antibodies against HLA-G (12).

HLA-G is expressed in vivo by all populations of *extravillous cytotrophoblast* cells during the first trimester: invasive extravillous cytotrophoblast, including interstitial cells (which invade the decidua basalis), endovascular cells (which penetrate maternal spiral arteries) and placental bed giant cells (8, 13-18).

HLA-G is also expressed in the endothelial cells of *fetal vessels and capillaries* present in the mesenchymal core of chorionic villi and within the chorionic plate (16).

HLA-G1 was detected on purified *amniotic cells* (19) and a recent study has detected soluble HLA-G in *amniotic fluid* (14, 20).

Placental *macrophages* present in the mesenchymal core of chorionic villi of first trimester placenta, also express HLA-G protein (16).

All of the **functions** of HLA-G during pregnancy have not yet been established, however there are some data about the different properties of the HLA-G protein. The recent results suggest three main possible functions of HLA-G.

The first function that HLA-G might have; is inducing *resistance to lysis by decidual NK (natural killer) cells expressing KIR (killer inhibitory receptor) that recognizes HLA-G.* There are already few KIR receptors described, which are able to recognize HLA-G. The first receptor is LIR1/ILT2. It is expressed in all cell types involved in the immune response including NK cells, T-cell subsets, B-cells and myelomonocytic cells (21,22). Another HLA-G recognizing receptor is ILT4, expressed on human macrophages, monocytes and dendritic cells (23). The third inhibitory receptor designated p49, also recognizes HLA-G (24). Finally, a new receptor designated BY55, was shown recently to interact with HLA-G, in addition to HLA class Ia and HLA-E (25). The effects of HLA-G/BY55 ligation on the potential target cells are still unknown. It cannot be excluded that other unknown

receptors recognize HLA-G. Recent studies have demonstrated that the ligand of CD94/NKG2A was in fact HLA-E and not HLA-G (22, 26-28).

There is evidence now, that HLA-G plays a role in the *regulation of HLA-E expression* (29). Regulation of HLA-E at the cell surface of extravillous cytotrophoblast , is therefore likely to be dependent on HLA-G biosynthesis.

The next potential function that HLA-G expressed on extravillous cytotrophoblast could exert, upon ligation to KIR receptors, would be the *release of particular cytokines* by these NK cells (30). In vitro experiments have shown that co-culture of human peripheral mononuclear blood cells with HLA-G-expressing cells resulted in a Th2 (T helper 2) response, as evidenced by an increased secretion of IL 3 and IL 1β and decreased release of tumour necrosis factor-α (TNFα) (31).

The third main function that HLA-G is likely to exert at the feto-maternal intreface is its *anti-viral function*. HLA-G binds nonamer peptides (32,33). There is evidence that it is capable of binding peptides of self origin (34), and it is likely to bind peptides of viral origin. Viruses that commonly infect the placenta are limited in number (35). Therefore, the low diversity of peptides presented by HLA-G may be sufficient to play a critical role in the presentation of viral peptides to cytotoxic T cells in the placenta (12).

In addition an interaction between CD8 and HLA-G has been demonstrated (36). Soluble HLA-G may cause *anergy and apoptosis of allogeneic cytotoxic CD8+ T cells* (37,38) and has the capability of *regulating placental angiogenesis* (16).

The other important MHC class Ib molecule is **HLA-E**. All of the class Ib genes are located on the same 6p21.3 chromosomal region and exhibit similar properties such as a limited **polymorphism** (only two alleles of HLA-E locus have been described) (39).

Human class Ib genes, compared with class Ia genes, are expressed in lower **amounts** and their **tissue distribution** is more restricted, at least at the protein level. The HLA-E gene itself seems to be ubiquitous. Until recently, the lack of HLA-E specific reagents made the in vivo study of HLA-E-cell-surface expression more difficult. In vivo, human HLA-E membrane-bound proteins were only detected in amnion epithelial cells by the microsequencing of the N-terminal amino acid sequence of an 41 kDa class I product, that was isolated from an individual amnion preparation (40). However A. King *et al.* demonstrated HLA-E expression in trophoblast by immunohistochemistry (personal communication).

Taken together, the above data suggest that HLA-E may play a role during human pregnancy. However, the importance and **function** of HLA-E during pregnancy is still under investigation. It has been reported that NK cells can interact with HLA-E complexed with specific peptides on target cells and that this recognition is mediated, at least partially, if not solely, by the CD94/NKG2 receptor (26).

Recent data reveled that only the HLA-G1 isoform was expressed as a membrane-bound protein at the cell surface of transfected cells. The intracellular retention of the three shorter forms of HLA-G (HLA-G2,-G3 and -G4) suggest that they may play a role in regulating cell surface expression either of the full length HLA-G1 form or HLA-E (41). The leader sequence of HLA-G contains a nonamer capable of stabilizing HLA-E resulting in a coexpression of HLA-E, in addition to HLA-G, on the cell surface. Based on these data, it has been proposed that HLA-G and HLA-E form multimeric complexes on the cell surface (22) and that HLA-G plays a role in the regulation of HLA-E expression (29,42).

2. GENERAL OVERVIEW ON HUMAN NK CELLS AND NK CELL RECEPTORS

Cytolytic T lymphocytes are representatives of the adaptive immune system and they are primed by specific exposure to an antigenic peptide bound in association with cell surface MHC molecules. NK cells belong to the system of innate (natural) immunity and for cytolytic activity do not require former antigenic stimulation. NK cells are defined as large granular lymphocytes, which usually express CD16 and/or CD56 in human and do not express CD3 or any known T-cell receptors (43).

NK cells may kill target cells by utilizing both secretory (perforin/granzyme-mediated) and nonsecretory (cell membrane-bound Fas ligand-mediated) mechanisms (44,45). Perforin is a pore forming protein, a cytolytic molecule, located in granules together with granzymes-serine esterases. Fas ligand was found to be expressed on the cell surface of NK cells and is responsible for Fas-mediated cytotoxicity against Fas-expressing target cells (45).

The NK cell lysis is non-MHC-restricted, furthermore NK cells are capable of secreting different cytokines (46-49).

The molecular mechanisms by which NK cells lyse or fail to lyse target cells have been intensively studied. NK cells express receptors that recognize MHC class I molecules on target cells. According to the **"Missing Self" hypothesis**, one function of NK cells is to recognize and eliminate cells that fail to express self MHC class I molecules (50). Thus, NK cells look for the absence of class I molecules, a common consequence of virus infection and malignant transformation.

The specific recognition of self-MHC class I molecules on target cells upon NK cell receptors may repress cytotoxic function of NK cells. Accordingly, cytotoxicity may occur if target cells lose MHC class I expression, if NK cells lack the appropriate killer inhibitory receptor or if there are changes in the structure of the peptide-MHC molecule impair recognition. Inhibitory NK receptors are also expressed on a small subset of human T lymphocytes (51).

Killer inhibitory receptors can be classified in two types. The first type include *members of the Ig superfamily.*

p58.1 and p58.2 function as receptors for two groups of HLA-C alleles (10). p70 functions as a receptor for the HLA-B alleles belonging to the Bw4 supertypic specificity (52,53). p140, on the other hand, recognizes some HLA-A alleles including –A3 and –A11 (54,55).

The second type of NK inhibitory receptors is represented by the molecular complex involving CD94 (56), a *member of the C-type lectin family* (57).

When associated to NKG2 molecules, CD94 forms an HLA class I specific inhibitory receptor, in that it recognizes different HLA-A, HLA-B and HLA-C allotypes (58-61).

The need for inhibitory NK cell receptor to prevent NK-mediated lysis of self cells implies the existence of **killer activating receptor**s that interact with ligand(s) expressed on target cells. However, limited information is available on the surface receptors that are involved in NK cell triggering. A molecule, termed *p46*, has been identified that induces strong NK cell activation upon cross-linking by specific mAbs (monoclonal antibodies). The p46 molecule is selectively expressed on all human NK cells, resting or activated, and the levels of cytolytic activity of different NK clones against HLA-negative targets correlates with the number of p46 molecules

expressed at their cell surface. p46 is clearly distinguishable from *p50* molecules (i.e., p50.1, p50.2 and p50.3, the HLA-C specific activating receptors) because of its molecular size, cell distribution, and distinct specificity. p46 may represent a receptor for non-HLA ligands expressed on both HLA^+ and HLA^- target cells (62).

On the other hand, in some NK clones, CD94 can mediate NK cell activation rather than inhibition (57,61). In this case, CD94 associates with NKG2C molecule which is identical with Kp39, to form an activating receptor complex named CD94/NKG2C (63,64).

The lack of a harmful NK activity to the trophoblast during normal pregnancy is due to the following mechanisms:

1. The effect of Progesterone Induced Blocking Factor (*PIBF*) resulting in inhibition of NK activity via blocking IL-12 production (65) and via blocking of NK cell degranulation and perforin liberation (66).

2. *Th2 shift*, involving reduced IL-12 production (67).

3. The presence of *HLA-G* (and probably HLA-E) on the trophoblast, which provides protection against NK activity partly due to recognition of the above-mentioned self MHC class I molecules via killer inhibitory receptors (22), partly due to inhibitory effect of HLA-G on transendothelial migration of NK cells, by which HLA-G may inhibit NK cell traffic across the placenta (68).

3. CHARACTERISTICS OF γ/δ T CELL RECEPTOR + LYMPHOCYTES

Most T cells recognize antigen via TCR αβ-CD3 complex on the T cell surface. A small percentage of T cells, however, do not express αβ but a second type of TCR complex designated γδ. This minor population represents a really low percentage of peripheral lymphocytes (1-10%) (69-75).

α/β T cells responsible for effective cell mediated immunity are essential for protection against invading pathogens. These cells often recognize processed antigens as short peptides presented on the cell surface of antigen-presenting-cells bound to MHC (76,77). On the other hand γδ T cells recognize a distinct group of ligands from αβ cells therefore play a different role in immunity. γ/δ T cells, in contrast to α/β, do not essentially require the presence of classical polymorphic or nonpolymorphic MHC molecules for a rapid and potent proliferation and stimulation (78,79). It is possible that most γδ cells recognize unprocessed foreign antigen without contribution of MHC and that γδ cells are able to recognize a wide set of different ligands. These ligands could be single-molecule ligands, rather than peptides, bound to larger molecules *(Fig.2.)*. All of the mechanisms and ligands recognized by γ/δ T cells are still unclear.

There are many data available about heat shock proteins as potent ligands for γδ T cells. *Heat shock proteins* (HSPs) are a highly conserved group of proteins widely distributed in nature (80). Increased synthesis of these proteins occurs in response to different forms of environmental stress, including temperature changes, inflammation, fever, irradiation, viral infection, malignant transformation, exposure

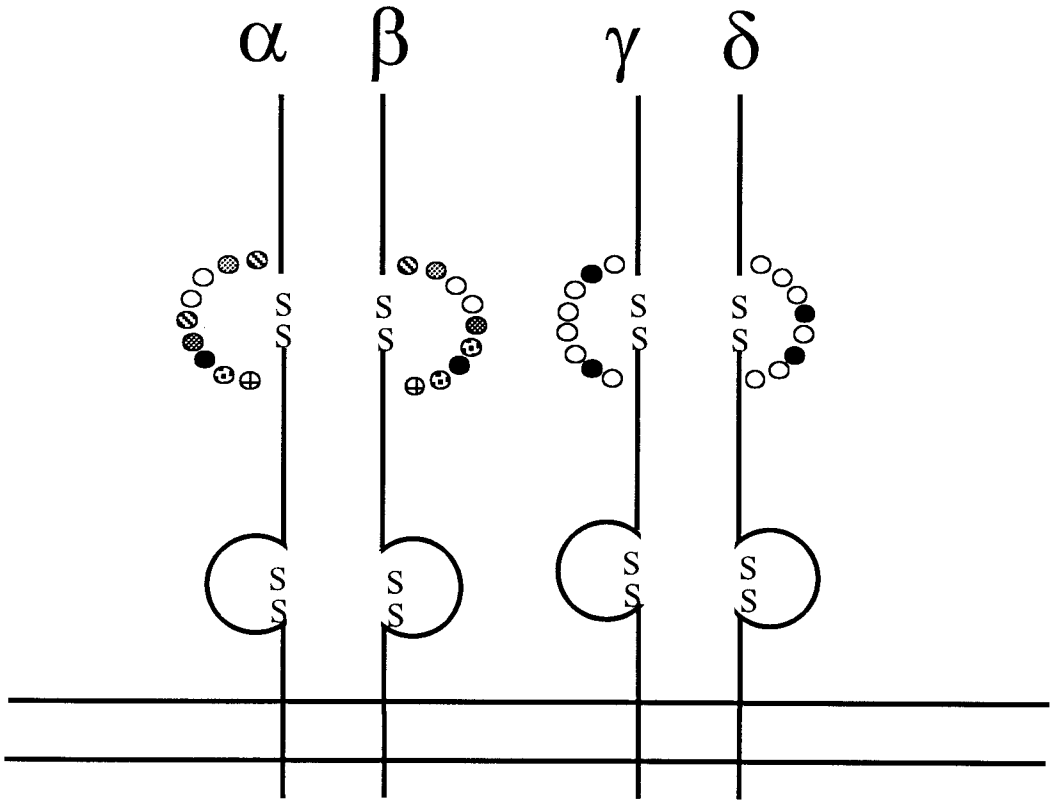


Fig.2. *The structure of α/β and γ/δ TCR*

The V region of the γ/δ TCR is less variable in structure than its α/β TCR counterpart, consistent with the idea that they interact with highly conserved structures.

to oxidizing agents, heavy metal ions, ethanol, anoxia (80). T lymphocyte activation (81) or cellular differentiation (82) also result in increased HSP expression.

Haregewoin *et al.* (83) has isolated a human HSP65 reactive γδ cell line that apparently requires antigen presentation in the context of self. In contrast to that Born *et al.* (84) has demonstrated mouse γδ cells, reactive with HSP-65, which do not require polymorphic self-MHC molecules for antigen presentation.

A number of studies imply that γδ cells with specificities for the non-polymorphic class Ib molecules are far more common than cells that can recognize polymorphic MHC class Ia or class II (85-87). Class Ib molecules are therefore the most likely presenting molecules for HSP-derived or other γδ antigens.

Now it is widely accepted that γδ T cells could recognize *phosphorylated nonpeptidic ligands*, which are quite different from lipids, superantigens or calssical peptides that trigger αβ T cells. Biochemical analysis characterized the bacterial ligands for Vγ9/Vδ2 T cells as a small protease resistant molecules (88), with critical phosphate residues (89,90). In addition, there are phosphorylated synthetic non-natural products that are not present in living organism, nevertheless are able to stimulate Vγ9/Vδ2 T cells.

In the experiments it was not possible to select clones specific for only one ligand, due to a phenomenon named a *broad crossreactivity* (91). In spite of the broad crossreactivity, these cells are highly specific as far as the ligand structure is concerned, since both the number and position of the phosphate groups, as well as the type of carbon backbone are important for T cell activation (92).

There are two main populations in human, which represent the 70 to 90% of the γδ T cells in the intestinal epithelium (93,94). The Vγ9/Vδ2 subset, predominant in circulation responses to bacterial infections by recognizing soluble nonpeptide

antigens (95-98). The other subset is defined by expression of Vδ1 chain, however its function and ligands are not so well established. It is demonstrated that Vδ1+ subset may be heat shock protein reactive (84,99). Heyborne *et al.* reported that maternally derived γδ T cells are present at the maternal-fetal interface where the percentage of γδ T cells is enriched three- to fourfold compared with maternal spleen and twofold compared with nonpregnant uteri (100). The Vδ1+ subpopulation mediates trophoblast recognition in mice, in a non-MHC-restricted way (101).

Concerning the hypothesis, described by De Libero (89) Vγ9/Vδ2 T cells, the most abundant population of T cells in the peripheral organs of adults (74,91,102-104) are characterized as broadly reactive human T cells, which may have a sentinel function.

3.1 EXPRESSION OF NATURAL KILLER CELL RECEPTORS ON γδ TCR+ CELLS

Characteristic feature of the Vγ9Vδ2 TCR+ cell is its ability to respond to nonprocessed and nonpeptidic phosphoantigens in an HLA-unrestricted manner (92,97,98,105,106).

Functionally mature Vγ9Vδ2 T cells display a potent cytotoxic activity against tumor cells (78,105,107,108) or virus-infected targets (109-113) and, similarly to NK cells, express the receptors for HLA class I molecules (114-116).

Expression of CD94 was found on the majority of circulating human γδ T cell receptor positive cells. The expression of CD94 is considerably higher (~80%) than that found in αβ TCR+ cells (~4,2% from the same donor) and is closer to that found on NK cells, among which virtually all express CD94 (117-119). The inhibitory form

of CD94 receptor is found within the $CD94^{bright}$ population whereas the stimulatory receptor is found within the $CD94^{dull}$ population (60,117,120,121). It has been demonstrated that most peripheral γδ T cells preferentially express the inhibitory form of CD94 (122).

In contrast to most circulating αβ TCR+ cells, in which NK cell receptor expression is relatively rare, most circulating γδ T cells in human express at least one type of NK cell receptor and many express more than one. This suggests that the regulation of γδ T cell function is likely to be different from that found in most αβ T cells, involving activation (or inhibition) by signaling trough both the TCR and the NK cell receptor (122).

Vγ9Vδ2 T cells indeed express the inhibitory CD94/NKG2A receptor for HLA class I molecules. The anti-CD94 mAb inhibits Vγ9Vδ2 T cell proliferation in response to mycobacterial phosphoantigens (122) and also the HIV-induced Vγ9Vδ2 T cell expansion (122). Vγ9Vδ2 T cells stimulated with nonpeptidic mycobacterial antigens produce IFNγ and TNFα. Signaling through the CD94 receptor results in an inhibition of cytokine synthesis in peripheral Vδ2 T cells. Treatment with an anti-CD94 mAb blocks IFNγ and TNFα production of Vδ2 cells (123).

Eighty % of circulating γδ T cells express CD94 (118,119,124), the ligand of the nonclassical HLA Ib molecule, HLA-E on the cell surface (26). The simultaneous presence of broadly crossreactive T cell receptors and HLA class I receptors on Vγ9Vδ2 T cells involves coordinated interaction of stimulatory (CD3-TCR) and inhibitory (CD94-NKG2A) receptors with their ligands. As already described before, activated Vγ9Vδ2 T cells produce mainly two Th1 cytokines, i.e. tumor necrosis factor α (TNFα), interferon γ (INFγ) and lyse the target cells through perforin

mediated cytotoxicity. CD94 downmodulates the activation of γδ TCR induced by phosphorylated metabolites. CD94 engagement has a major effects on TCR signaling cascade (125). Moreover, most CD94 negative Vγ9Vδ2 T cells derived from peripheral blood express an intracellular pool of CD94/NKG2A receptor that is translocated to the cell surface upon activation by phosphoantigens (126).

All these data indicate that the regulation of γδ T cell function is likely to be different from that found in αβ T cells, involving activation (or inhibition) by signaling through both the T cell receptor and the NK cell receptor. γδ T cells could be the first-line of defense, complementing the αβ T cell and B cell responses, which may take more time to develop. This concept is supported by the observation that in rodents γ/δ T cells are preferentially localized in epithelial tissues; in the skin (127) and in the intestine (128,129), thus they could efficiently represent the first level of immune-protection against infectious agents (130,131).

The human Vγ9/Vδ2 T-cell subset appears to be of special importance, because it responds to both naturally occurring (particularly in microorganisms) and synthetic nonpeptidic phosphoantigens (91).

4. PROGESTERONE DEPENDENT IMMUNOMODULATION

Progesterone is essential for the maintenance of pregnancy. High concentrations of progesterone prolong the survival of xenogeneic and allogeneic grafts. (132,133) Progesterone inhibits the contractions of myometrial smooth muscle (134), blocks the activity of uterine collagenase (135) modifies the activity of proteolytic enzymes and has immunomodulatory properties. At the materno-fetal interface there is a high local concentration of progesterone (3-10 g/mg of placental tissue) as a result of local synthesis. The serum concentrations in mice and humans, however, are much too low to support the concept of a generalized immunosuppresion (136).

Lymphocytes of pregnant women develop progesterone receptors (PRs) (137). Earlier data from our laboratory suggest that PRs appear following lymphocyte activation (137,138,139). The existence of specific progesterone receptors in pregnancy, but not in non-pregnancy lymphocytes was demonstrated by our group (140). At the same time, significantly lower percentages of receptor-containing cells were found on lymphocytes of women at labour as well as on those of women with threatened pre-term delivery or with idiopathic spontaneous abortion compared with those of normal pregnancy (141).

Blocking of progesterone binding sites by progesterone receptor antagonists causes abortion (142,143).

In the presence of progesterone, PR positive lymphocytes produce a 34 kDa protein named the progesterone-induced blocking factor (PIBF) (144), mediating immunological effects of progesterone (145). PIBF has pleiotropic immunomodulatory properties, which are briefly the following:

- PIBF blocks arachidonic acid release from mononuclear cells, thus inhibiting prostaglandin synthesis (146), which has a major role in the initiation of labor. Our recent data revealed that blocking the metabolism of arachidonic acid by PIBF results in a decreased expression of IL-12 (147).

-Our group also demonstrated that PIBF inhibits NK activity by reducing IL-12 production (148) and by inhibition of NK-cell-degranulation (66).

- Via inhibition of NK activity, PIBF displays an anti-abortive effect in mice (149-153).

- PIBF alters the Th1/Th2 balance by inducing an increased production of IL-10, IL-3 and IL-4 when spleen cells are cultured with the progesterone-dependent protein (154). This altered cytokine ratio contributes to decreased cell-mediated responses and increased antibody production during pregnancy. Besides influencing the rate of antibody synthesis PIBF acts on the quality of antibody production.

- PIBF enhances asymmetric antibody production of hybridoma cells (155). Asymmetric antibodies have a mannose-rich olygosaccharide linked to one of the Fab arms of the molecule and though they bind the antigen with the same specificity as this conventional conterparts, these Ig molecules are generally unable to activate effector functions, such as complement fixation, phagocytosis and cytotoxicity, however, they can block the antigen and thus might play a role in the protection of the fetus. The sera of pregnant mice treated with anti-PIBF antibody contained four times less asymmetric antibodies than those of normal pregnant animals (156).

PIBF can be detected in sera of pregnant women and its concentration is higher than that of non-pregnant individuals or pregnant women with symptoms of threatened abortion. Similar results were obtained by testing PIBF expression on lymphocytes of healthy pregnant women and those of women with pathological pregnancy (157).

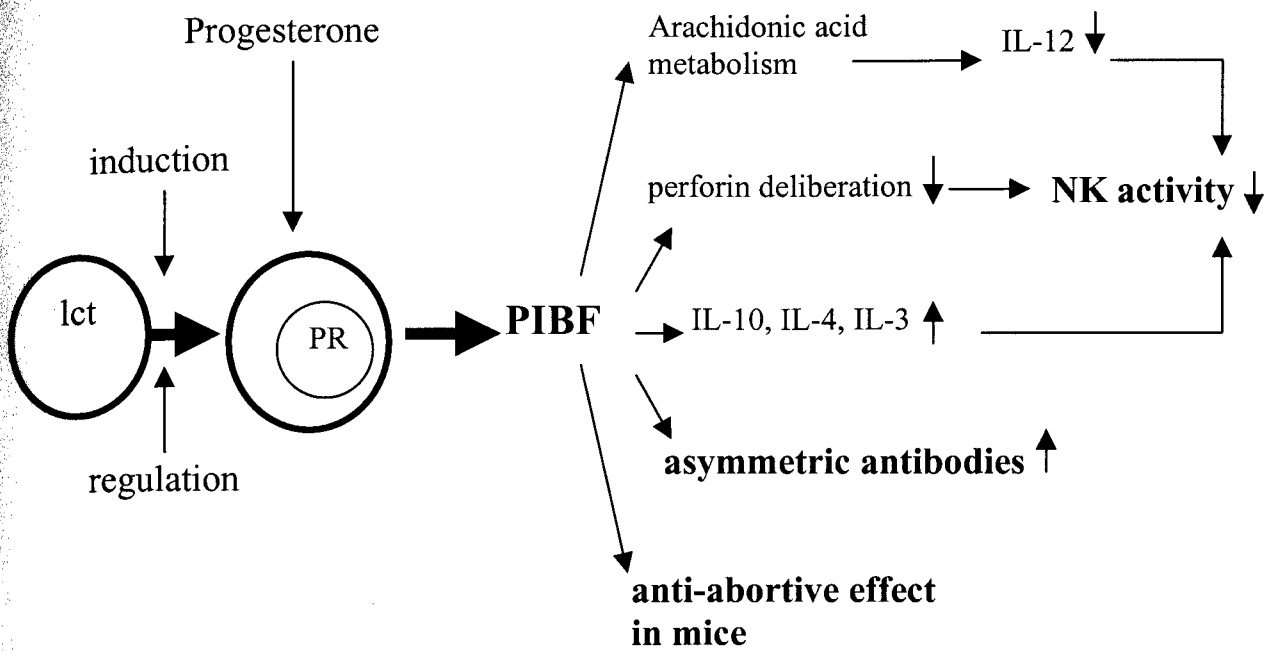


Fig.3.: Progesterone-dependent immunomodulation during pregnancy

The schematic representation of the progesterone-dependent immunomodulatory pathway is shown in *Fig.3.*

Normal pregnancy lymphocytes express specific binding sites for progesterone. This allows the production of the blocking factor when lymphocytes are exposed to the hormone. PIBF initiates immunological events leading to an impairment of NK and cytotoxic activity that favors a normal pregnancy outcome.

III. AIMS OF THE STUDY AND RESULTS

1. The possible role of γ/δ TCR+ cells in progesterone-dependent immunomodulation during normal and pathological pregnancies (Paper 1)

Due to lack of classical HLA antigens on the trophoblast, fetal antigens are possibly presented in a non-MHC-restricted way. There is an increased presence of γ/δ T cells in the decidua (158) and these decidual γ/δ T cells preferentially use the Vδ1 chain. In contrast to this, among γ/δ lymphocytes in peripheral blood of healthy individuals Vδ2 chain is predominant (159). Decidual γ/δ T cells may play a role in recognition of fetal antigens. Our study was aimed at investigating the possible involvement of this cell population in progesterone dependent immunomodulation.

In peripheral blood of healthy pregnant women the percentage of γ/δ TCR+ cells was significantly higher than in that of recurrent aborters or of non-pregnant individuals. Ninety-seven percent of γ/δ TCR+ peripheral pregnancy lymphocytes were progesterone receptor (PR) positive at the same time.

Almost all decidual γ/δ T cells are in an activated form (160), thus it is possible, that following recognition of fetal antigens on the trophoblast these lymphocytes develop PRs and appear at the periphery.

Earlier we demonstrated a reduced percentage of PR (progesterone receptor) positive lymphocytes in peripheral blood of recurrent aborters (139). In line with these observations, in peripheral blood of recurrent aborters we found a significantly lower ratio of PR and γ/δ TCR double positive lymphocytes; however, still 83% of all γ/δ positive cells expressed progesterone receptor. In non-pregnant individuals, the total

number of γ/δ TCR positive cells was not significantly lower than that of recurrent aborters, only 14% of these expressed PR.

Among peripheral lymphocytes of healthy pregnant women the ratio of IL-10 positive γ/δ T cells was significantly higher than in that of recurrent aborters. In both groups γ/δ T cells constituted a high proportion of all IL-10 positive cells, suggesting that in normal pregnancy γ/δ T cells might contribute to the Th2 response.

Treatment of lymphocytes with anti-pan-γ/δ antibody inhibited PIBF as well as IL-10 production and raised NK activity and IL-12 production. Pan-anti-γ/δ antibody recognizes all subsets of the γ/δ population and the final effect depends on the proportion of the functionally different subsets.

These data suggest that γ/δ T cells might play a part in progesterone-dependent immunomodulation. It is possible that γ/δ T cells recognize fetally derived antigens at the feto-maternal interface, become activated and develop progesterone receptor. These progesterone receptor positive T cells in the presence of progesterone produce PIBF, contributing to the Th2-shift (161) and decreasing local cytotoxicity on the trophoblast, which allows the semmiallogenic fetus to survive.

2. *V-chain usage and function of γ/δ subpopulations in peripheral blood of healthy pregnant women and of pregnant recurrent aborters (Paper 2)*

The majority of human peripheral γ/δ T cells express the Vγ9/Vδ2 TCR, which is able to recognize nonprocessed and nonpeptidic phosphoantigens in an HLA-unrestricted manner (90,95,96,104,105). In contrast to those on the periphery, almost all γ/δ T cells in the decidua use the Vδ1 chain and only a small fraction uses the

Vδ2 chain (158). We investigated the presence and function of the different γ/δ subpopulations during normal and pathological pregnancies.

Our results show that in peripheral blood of healthy pregnant women the ratio of Vγ1.4/Vδ1 to Vγ9/Vδ2 cells was eight times higher than in that of recurrent aborters, thus is seems that normal pregnancy is characterized by an altered rate of these subpopulations, and these changes are absent in recurrent abortion.

Preincubation of normal pregnancy lymphocytes with anti-Vγ1.4 and anti-Vδ1 antibodies resulted in a significantly increased PR expression and IL-10 production as well as a reduced NK activity. This treatment exerted no significant effect on IL-12 production, whereas treatment with anti-Vγ9 and anti-Vδ2 antibodies resulted in a significantly decreased IL-10 production but did not influence significantly IL-12 production, NK activity or PR expression.

We have previously found a significantly increased NK activity after anti-pan-γ/δ treatment of pregnancy lymphocytes. Although Vγ1.4 and Vδ1 populations increase during normal pregnancy, they still represent minor subsets of the whole γ/δ T cell population. Thus it is likely that anti-pan-γ/δ antibody, acting on all subpopulations resulted in an increased cytotoxicity.

Based on our findings we developed the following hypothesis: Vγ1.4/Vδ1 T cells predominant in decidua could recognize antigens presented by trophoblast. Following antigen recognition these cells become activated and develop PRs, which enables them to produce PIBF in the presence of progesterone. PIBF inhibits NK activity, increases IL-10 production, and thus contributes to normal pregnancy outcome.

3. Recognition of nonclassical HLA antigens by γ/δ T cells during pregnancy (Paper 3)

Since trophoblast is known as a tissue expressing nonclassical MHC molecules (8) and there is an increased presence of γ/δ T cells in the decidua (131) it can not be excluded that these nonclassical MHC molecules may act as ligands of γ/δ T cells on the trophoblast. Following recognition of these structures, γ/δ T cells might become activated or inhibited. This study was aimed at investigating the possible recognition of nonclassical MHC molecules by γ/δ T cells from healthy and pathological pregnant women and its consequences.

For recognizing cell surface associated antigens lymphocytes need to get in close contact with the antigen-expressing cell. Therefore, conjugation of lymphocytes to target cell reflects a component of antigen recognition. After adhesion lymphocytes could be activated or inhibited.

In this study we used the choriocarcinoma cell line JAR and its HLA transfected form, JAR-G. JAR-G was transfected with the HLA-G gene, which contains a nonamer capable of coexpressing HLA-E in addition to HLA-G on the cell surface. Two other transfectants, i.e., JAR expressing HLA-E (JAR-E), and that expressing HLA-G only (JAR-G1m) were used as controls.

We have found a significantly increased conjugation capacity of peripheral healthy pregnancy lymphocytes to HLA class I negative JAR cells compared to that of peripheral pan-γ/δ T cells, which preferentially conjugated to JAR-G. These observations suggest that α/β T cells of pregnant women in contrast to γ/δ T cells do not recognize non-classical HLA antigens, but other structures presented by the trophoblast.

We have determined the conjugation capacity of four different γ/δ subsets: the Vγ9, Vδ2, Vγ1.4 (also known as Vγ4) and Vδ1 positive ones. Our results showed a significantly increased conjugation of the Vδ2+ population to choriocarcinoma cells expressing HLA-E. The other subsets being investigated did not show any altered conjugation capacity to the different target cells. In contrast to normal pregnancy Vδ2+ cells, Vδ2+ lymphocytes derived from peripheral blood of pregnant women at risk for premature pregnancy termination did not show significant difference in the rate of conjugation to the HLA negative or HLA-G or -E transfected choriocarcinoma cells.

We assumed that CD94/NKG2A complex, the known killer inhibitory receptor, which is also expressed on γ/δ T cells (116-118) could be involved in the increased recognition of JAR-G and JAR-E cells by healthy pregnancy Vδ2+ cells. To explore this possibility, magnetic bead separated Vδ2+ cells from healthy pregnant women were incubated with a blocking concentration of anti-CD94 antibody and then conjugated to the different target cells. Blocking of the CD94 molecule inhibited conjugation of Vδ2+ cells to JAR-G.

These results revealed that γ/δ T cells preferentially recognize target cells, which express nonclassical MHC antigens. Within the γ/δ T cell population, lymphocytes bearing Vδ2 chain could recognize HLA-E, which might be coexpressed in HLA-G transfected JAR cell line. Our recent data obtained with HLA-E transfectants and JAR expressing HLA-G without HLA-E confirmed this hypothesis.

Signalling through the CD94 receptor expressed on peripheral Vδ2+ T cells induces an inhibitory signal for Th1 type-cytokine synthesis (122), contributing to normal pregnancy. Our findings suggest an additional function of HLA-E on the trophoblast. Upon recognition of nonclassical MHC molecules via CD94 the potentially cytotoxic

Vγ9/Vδ2 T cell population is inhibited. Since in contrast to Vδ2+ cells from healthy pregnant women, those of pathological pregnant women did not show an increased capability to conjugate with JAR-G, it cannot be ruled out that in pathological pregnancy nonclassical MHC does not act as a ligand for CD94/KIR receptor. This might result in high (uninhibited) cytotoxic activity, contributing to premature termination of pregnancy.

IV. THESES

1. In peripheral blood of healthy pregnant women the percentage of γ/δ TCR+ cells was significantly higher than in that of recurrent aborters or of non-pregnant individuals. The consistent presence of progesterone receptor expression on healthy pregnancy γ/δ TCR+ suggest that these γ/δ cells are activated.

2. Among peripheral blood lymphocytes of healthy pregnant women the ratio of IL-10 positive γ/δ T cells was significantly higher than in that of recurrent aborters.

3. Treatment of lymphocytes with anti-pan-γ/δ antibody inhibited PIBF as well as IL-10 production and raised NK activity together with IL-12 production. Progesterone receptor positive γ/δ T cells in the presence of progesterone produce PIBF. PIBF induces a Th2-shift, thisway reducing local cytotoxicity at the feto-maternal interface, which allows the semmiallogenic fetus to survive.

4. In peripheral blood of healthy pregnant women, the most frequently occurring chain combination was Vγ1.4/Vδ1, whereas in recurrent aborters, the Vγ9/Vδ2 combination was predominant.

5. Activation of γ/δ T cells via Vγ1.4/Vδ1 T cell receptor resulted in a significantly increased progesterone receptor expression, reduced NK activity and increased IL-10 production. On the other hand triggering of Vγ9/Vδ2 T cell receptor exerted no effect on PR expression, NK activity and IL-12 production, but

resulted in a significantly reduced IL-10 production. These results suggest the presence of two functionally distinct subpopulations in peripheral blood, the cytotoxic γ9/δ2 population and the γ1.4/δ1 population, which acts in a Th2 like manner.

6. The rate of peripheral blood lymphocytes conjugated to HLA negative choriocarcinoma JAR cells was significantly higher than that to JAR-G suggesting that peripheral pregnancy lymphocytes do not recognize HLA-G and/or HLA-E, but other structures on the trophoblast.

7. Pan-γ/δ TCR+ cells conjugated preferentially to JAR-G cells suggesting that they do recognize nonclassical HLA.

8. γ/δ T cells expressing the Vδ2 chain, similarly to pan-γ/δ TCR+ ones show a significantly increased rate of conjugation to JAR-G or JAR-E versus JAR cells, whereas Vγ9, Vγ1.4 and Vδ1 chain positive lymphocytes do not discriminate between the two cell lines.

9. Vδ2+ lymphocytes recognize HLA-E via a killer inhibitory receptor CD94 and this interaction could deliver an inhibitory signal for cytotoxic activity, contributing to the normal pregnancy outcome.

10. Peripheral Vδ2 + cells from pathological pregnant women do not recognize HLA-E, resulting in a lack of inhibition of cytotoxicitiy. This may play a part in

the inadequate maternal antifetal immune-response observed in failed pregnancies.

V. APPENDIX (METHODS IN THE STUDY)

1. Separation of peripheral blood lymphocytes

Lymphocytes were isolated from heparinised peripheral blood on Ficoll-Paque gradient, washed twice with RPMI 1640 medium and adjusted to a cell count of $1x10^6$/ml. The cells were incubated with appropriate concentrations of different monoclonal antibodies or conjugated to target cells.

2. Monoclonal Antibodies

The following monoclonal antibodies (mAbs) were used: mouse anti-human PR (Immunotech, Marseille, France), mouse anti-human TCR γ1.4 (Serotec, Oxford, UK), murine mAb to human TCR Vδ2, Vδ1, Vγ9 and pan γδ (T-cell Diagnostic Inc., Woburn, MA, USA), mouse anti-human TCR Vδ2-FITC (Pharmingen, San Diega, CA, USA), mouse anti-human IL-10 and IL-12 (R&D System, Minneapolis, MN), mouse anti-human CD94 (Serotec, Oxford, UK) and mouse IgG2a, kappa, isotype control (Sigma, Saint Louis, MO, USA)

3. MiniMACS γδ T cell separation

Vδ2+ T cells were separated using MiniMACS immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, cells were washed with phosphate-buffered saline (PBS and resuspended at $1x10^7$ / ml PBS containing 0,5% bovine serum albumin (BSA) and 2 mM EDTA. Cells were incubated for 10 minutes at 4°C with 10 μg anti-Vδ2 mAb per 10^7 cells. After incubation, 10^7 lymphocytes were washed twice and resuspended in 80 μl of buffer and 20 μl of Goat anti-Mouse

IgG Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added. Cells were incubated for 15 minutes at 4°C and washed. Pelleted cells were resuspended in 500 µl buffer and applied to a MiniMACS column fitted to a magnet. The column was washed six times and then removed from the magnetic separator. The magnetic adherent cells were flushed out of the column in to a fresh tube using a plunger included in the MiniMACS Kit. The percentage of Vδ2 TCR+ cells adhering to the magnetic beads was about 75% of the total adherent cells.

4. Flow cytometry

For testing the purity of Vδ2+ population $1x10^5$ magnetically separated lymphocytes were washed in FACS (fluorescence-activated cell sorter) buffer and incubated with an appropriate dilution of FITC (fluorescein-isothiocyanate) conjugated anti-Vδ2 mAb for 30 minutes. During incubation, samples were protected from light. Then cells were washed with FACS buffer and resuspended in 250 µl FACS buffer containing 1% paraformaldehyde and stored at 4°C, in dark to be processed for FACS analysis the next day. Samples were analyzed on a FACScan flow cytometer using the Lysis II software.

5. Treatment of lymphocytes or magnetically separated Vδ2 TCR+ cells

Peripheral blood lymphocytes and magnetically separated Vδ2 TCR+ cells were incubated with appropriate concentrations of mAbs at 37°C (5µg anti-pan-γ/δ mAb/ml/$1x10^6$ lymphocytes, 1µg anti-Vγ9, anti-Vδ2, anti V-γ1.4, anti-Vδ1 mAbs/ml/$1x10^6$ lymphocytes: for 3 hour and 10µg anti-CD94 mAb/ml/$1x10^6$ lymphocytes for 30 minutes). After incubation cells were washed in medium.

Untreated samples and those reacted with irrelevant antibody of the same isotype were used as controls.

6. Conjugation of lymphocytes or magnetically separated Vδ2 TCR+ cells

The ratio of target cells to lymphocytes (or magnetic bead separated Vδ2 TCR+ cells) was 1:10. The mixture of cells was centrifuged at 500 rpm for 5 minutes and incubated at 37°C in 5% CO_2 for 10 minutes. After conjugation the cells were resuspended and centrifuged on glass microscope slides. The slides were dried at room temperature and fixed in cold acetone for 5 minutes. The proportion of the different γδ subpopulations bound to target cells was investigated by immunocytochemistry.

In case of magnetic bead separated Vδ2 TCR+ cells after treatment and conjugation, slides were stained with Hematoxilin-eosin.

7. Immunocytochemistry

Endogenous peroxidase activity of the cells was blocked with 1% H_2O_2. Nonspecific protein binding sites were blocked by 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO). All incubations were carried out at room temperature in a humid chamber. The primary antibodies (monoclonal anti-γδ subsets, anti-progesterone receptor, anti IL-10 and anti IL-12) were diluted 1:50 in Tris-buffered saline (TBS) containing 0.5% BSA. After 1 hour of incubation with the primary antibody, 1:100 diluted HRPO (horseradish peroxidase) labeled anti mouse immunoglobulin was added as a secondary antibody for 30 minutes. The slides were washed three times in TBS and the reaction was developed by diaminobenzidine and intensified with silver staining.

In all cases, control slides without the primary antibodies were included.

8. Four-Hour Single Cell Cytotoxicity Assay for NK activity

We used the assay originally described by Grimm and Bonavida (162). One hundred microliters of lymphocytes and the same amount of K562 target cells ($2x10^6$cells/ml each) was centrifuged at 500 rpm for 5 min and incubated at 37 °C, in 5% CO_2 for 10 min. The pallets were then resuspended and 200 µl of 1% agarose (Serva, Heidelberg, Germany) in RPMI 1640 was added to the mixture. Two hundred µl of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and submerged in RPMI 1640. The slides were incubated for 4 hr at 37°C in 5% CO_2. The gels were then stained with 0.5% trypan blue for 1 min. After 2-min washes with PBS, the gels were fixed in 2% formaldehyde for 5 min and desalted in distilled water. The slides were read using a light microscope with 400x magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 100 lymphocytes. Results are expressed as a percentage of target binding cells (TBC). Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates, and results are expressed as a percentage of dead conjugates (cytotoxic TBC%). The percentage of NK cells was calculated according to the formula NK%=(TBC% x cytotoxic TBC%)/ 100. All results for cytotoxic TBC% were corrected for the proportion of target cells that died spontaneously in control plates.

9. Statistics

The two-tailed Student's t-test and the Paired t-test were used for statistical evaluation of the data. Differences were considered significant if the *P* value was equal to or less than 0.05.

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VIII. PUBLICATIONS

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