Immunological Aspects of Maternal-Foetal Interactions in Mice

BY

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ABSTRACT


Mammalian pregnancy is an immunological paradox. The foetus, which expresses both paternal and maternal cell-surface molecules, has to be protected from rejection by the maternal immune system. At the same time, the mother has to have an efficient immune defence and must provide her offspring with antibodies.

The first part of this thesis investigates some of the mechanisms involved in the foetal avoidance of maternal rejection reactions. Placental absence of MHC class II expression, as well as a bias for Th2-cytokines at the maternal-foetal interface are suggested to be important for foetal survival. The results showed that placental MHC class II expression cannot be induced \textit{in vivo}. Transfections of trophoblast cells with MHC class II genes, however, resulted in detectable MHC class II cell-surface expression, indicating the capacity for functional class II mRNA and protein processing in these cells.

By using IL-4- and IL-10-double deficient mice, it was shown that neither maternal nor foetal expression of these cytokines were crucial for completion of allogeneic pregnancy.

In the second part of the thesis, the effect of transmission of immunoglobulin G (IgG) from the mother to the offspring was studied. It was observed that viable maternal IgG-secreting cells occasionally infiltrated the B cell-deficient offspring and remained functional for long periods. In this study "green fluorescent mice" were used as a tool. Furthermore, neonatal ingestion of wild type milk increased the survival of adoptively transferred B-lineage cells in B cell-deficient mice, suggesting that suckling of IgG-containing milk could be used to facilitate B cell-reconstitution in B cell-deficient mice. Finally, results from studies on normal mice showed that absence of maternal IgG-transmission during their neonatal development resulted in elevated serum-IgG production, as well as enhanced immune reactions upon immunisations in adult life. This showed that maternal IgG can have long-term immunoregulatory effects in the offspring.

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_Biology of Reproduction_ 1997; _57_: 715-722

II. Marie Arvola, Erika Gustafsson, Ulrica Brunsberg and Ragnar Mattsson. Human choriocarcinoma-derived JEG-3 cells transfected with murine MHC class II A\( q \) expression vectors present antigen to A\( q \)-restricted murine T-cell hybridoma. 
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III. Lars Svensson, Marie Arvola, Mary-Ann Sällström, Rikard Holmdahl and Ragnar Mattsson. The Th2 cytokines IL-4 and IL-10 are not crucial for the completion of allogeneic pregnancy in mice. 
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IV. Marie Arvola, Erika Gustafsson, Lars Svensson, Liselotte Jansson, Rikard Holmdahl, Birgitta Heyman, Masaru Okabe and Ragnar Mattsson. Immunoglobulin-secreting cells of maternal origin can be detected in B cell-deficient mice. 
_Biology of Reproduction_ 2000; _63_: 1817-1824

V. Marie Arvola, Erika Gustafsson and Ragnar Mattsson. Neonatal ingestion of IgG-containing milk increases the survival of adoptively transferred B-lineage cells in B cell-deficient mice. 
_Journal of Reproductive Immunology_ 2001, _In press_

VI. Marie Arvola, Erika Gustafsson and Ragnar Mattsson. Absence of maternal IgG-transmission causes elevated serum-IgG levels and enhanced immune responses in young C57BL/6 mice. 
_Manuscript_

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### ABBREVIATIONS

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<th>Description</th>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II transactivator</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunospot assay</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FcγR</td>
<td>Fc receptor for IgG</td>
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<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor for IgG</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>gd</td>
<td>Gestation day</td>
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<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
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<tr>
<td>Pro-B</td>
<td>B cell progenitor</td>
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<tr>
<td>Pro-T</td>
<td>T cell progenitor</td>
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<tr>
<td>SPF</td>
<td>Specified pathogen-free</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<td>T helper cell type 1</td>
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INTRODUCTION

The survival of an individual depends on several different factors, such as defence against predators and availability of food and water. One important factor, which may not be so obvious, is the protection against infections and parasites. In the body, the immune system has evolved to take care of this protection by distinguishing self from non-self and thereby recognising and removing foreign molecules.

In order to adapt the immune response to every foreign "intruder" of the body, the immune system shows an enormous flexibility and complexity in all aspects. Consequently, it consists of several different cell-types and components, which interact by intricate signalling pathways via both soluble molecules and direct cell-to-cell contact.

From an evolutionary point of view, not only is the survival of the individual important, but a successful reproductive strategy is also required; where the offspring is provided with the very best options for survival early in life, in order to reach the reproductive age. In mammals, the immune system of the mother contributes to the survival of the offspring in a very delicate way. The offspring is provided with antibodies from the mother via both placental transport and the breast milk, which will give protection against pathogens that are frequently found in the environment. The antibodies in the milk are important in the defence against parasites in the gastro-intestinal region of the neonate, but are also taken up into the bloodstream in some species, where they might influence the immune system. In fish and birds, the mother also contributes to passive immunity by transferring antibodies into the yolk of the egg, from where it is then transported into the embryo proper.

The studies included in this thesis deal with different aspects of immunological interactions between the mother and her offspring in mice and might shed some light onto several questions raised in this area. Some of the interactions discussed are involved in protecting the foetus from rejection by the maternal immune system in utero, while others are involved in the transfer of immunity to the neonatal mouse via milk.

THE IMMUNE SYSTEM

Immunology, the study of the immune system, originated from the common observation that people who recovered from certain diseases, did not get ill if they came in contact with the same infection later in life. In
other words, they became "immune" to that specific disease. This immunity is very specific, however, which means that if a person is immune to one infection, they might not be immune to other very similar infections. Such specificity is one of the characteristics of the immune system.

The mission of the immune system is to distinguish foreign molecules from self-molecules and clear the body from these foreign substances, which are known as "antigens". In order to perform this clearance and thereby keep the body free from infections, the immune system is comprised of several cell-types and components organised in a complex network. There are two broad categories into which the immune response to an antigen can be divided; innate immune responses and adaptive immune responses. Important differences between these types of responses are that an adaptive immune response is highly specific for a particular antigen, "remembers" the infectious agent and can also prevent it from causing disease later. The innate immune response, on the other hand, does not show any immunological memory and represents a more evolutionary conserved type of response.

The cells constituting the immune system originate from pleuripotent haemopoietic stem cells in the bone marrow and can be divided into two separate linages; the myeloid and the lymphoid lineage. Cells of the myeloid lineage are mainly involved in innate immune responses, whereas the lymphoid cells are the executors of the adaptive immune response. Other important parts of the immune system include the complement system and cytokines.

The myeloid lineage

The cells of the myeloid lineage include the monocytes, macrophages, granulocytes and mast cells. These cells are mainly involved in the innate immune response where, as the first line of defence, they exert powerful effector functions against bacteria and parasites, for example. There is also considerable interaction between the phagocytic cells of the myeloid lineage and the lymphocytes. For instance, phagocytes can take up antigen and present it to T lymphocytes and in turn, the T lymphocyte can release soluble factors, which can activate the phagocytic cell.
The lymphoid lineage

The lymphoid lineage gives rise to two major types of lymphocytes, the B cells and the T cells. One feature that B and T cells have in common is the ability of specifically recognising foreign antigens. Antigen recognition, which is the hallmark of the adaptive immune response, is totally dependent on the fact that these cells carry specific receptors on their surfaces, which vary among the cells in each population. The receptors on B cells are the membrane-bound immunoglobulins, while T cells carry so-called T cell receptors (TCRs). The enormous variation shown by the receptors is generated before the cells encounter antigen, which means that they can react not only towards foreign antigens, but also occasionally towards self-antigens.

The basis for the diversity of B and T cell receptors resides in the organisation of their genes. The variable regions of the receptors are encoded by multiple gene segments that rearrange during the development of the B and T cells. To eliminate the vast majority of self-reactive or inadequate cells, T cells with TCRs that bind either too strongly or weakly to major histocompatibility complex (MHC) molecules in the thymus are eliminated. In a similar way, B cells with receptors that react with self-antigens will undergo apoptosis. Occasionally some cells escape this modification of the receptor repertoire and might then contribute to the development of autoimmune disorders.

Both B and T cells are activated upon binding to their specific antigens. Antigen is presented to T cells by MHC molecules on antigen presenting cells (APCs). B cells on the other hand, can bind directly to free antigens, but generally need signals from T cells in order to become activated. The activated cells then constitute the effector cells of the adaptive specific immune response. In addition to B and T cells, the lymphoid precursor cells can also give rise to the natural killer (NK) cells and dendritic cells. The NK cells play an important role in, for example, killing of tumour cells.

B CELLS

B cell development

During development, the early B cell differentiation from haematopoietic precursor cells occurs at different locations. This differentiation is first observed in the splanchnopleure of the embryo (1), then in the foetal liver
and spleen (2) and finally in the adult bone marrow. The pleuripotent stem cells differentiate into mature cells of different lineages, including the B cell lineage.

The first phase of B cell-development is antigen-independent and involves a set of Ig gene rearrangements that first bring diversity (D) segments to joining (J) segments, whereupon variable (V) segments are attached, resulting in a functional Ig heavy chain gene (3). Prior to complete VDJ rearrangement, the cells are often referred to as pro-B cells, while later stages with the μ heavy chain protein bound to the surrogate light chains λ5 and VpreB, are called pre-B cells. Rearrangements of the V and J segments of the light chain result in the fusion of the Ig μ heavy chains with light chains to form a surface immunoglobulin on the immature or virgin B cell. The formation of the B cell receptor also requires the association with the signalling subunits Igα and Igβ, which are also present at the pre-B stage (4, 5).

During all these processes, unsuccessful rearrangements of the Ig genes will trigger the cell to undergo apoptosis. In the bone marrow, the B cells encounter membrane bound autoantigens and if they recognise such molecules, they will be depleted (6, 7) or become anergic (8). Expression of surface-IgM is crucial for B cell development, since mice with a targeted disruption of the µ chain gene lack endogenous production of functional B cells (9).

IgM- and IgD-expressing virgin B cells leave the bone marrow to circulate in the blood and home to different lymphoid tissues. If they do not encounter antigen, the cells will die within a short period of time, with a half-life of a couple of days. The antigen-activated B cells can respond to T cell-derived cytokines to switch constant chain production by gene rearrangements, resulting in antibodies of other isotypes and can further differentiate to either plasma cells or memory cells.

Plasma cells and memory cells

During T cell-dependent humoral immune responses, responding B cells differentiate into either memory B cells, or antibody-forming plasma cells, which are also called Ig-secreting cells. This lineage commitment requires molecular control via different signalling pathways. It has been reported that CD40-CD40 ligand, OX40-OX40-ligand, some interleukins (IL) and intracellular transcriptional factors may all contribute to the control of B-lymphocyte differentiation (reviewed in (10)). In brief, CD40 ligand, IL-4 and the transcription factor BSAP seem to inhibit plasma cell differentiation,
while OX40, IL-3, IL-6, IL-10 and Blimp-1 (B-lymphocyte-induced maturation protein-1) seem to promote plasma cell differentiation.

The memory cells circulate and home into certain areas in lymphoid organs, such as the germinal centres in the spleen, where they stay ready to respond if the same antigen is encountered again. In general, the lifespan of memory cells is very long, even without antigenic stimulation. Most memory B cells are thought to have undergone class switching, which increases their affinity for the antigen. When the memory cell encounters the same antigen again, it will rapidly produce a secondary immune response.

The plasma cells, on the other hand, are seldom seen in the circulation and are normally restricted to the lymphoid organs. One single plasma cell can secrete 10,000 Ig molecules per second (11, 12) and the antibodies produced are of the same immunoglobulin class, with the same specificity. During the differentiation, plasma cells lose some surface molecules, such as B220, MHC class II and surface-Ig and are unable to respond to regulatory signals from sources such as T cells.

Until quite recently, all plasma cells were thought to have a short life span, to ensure that the production of antibodies would not continue over an extended period of time. In recent years, however, it has been reported that some populations of plasma cells can survive for more than a year in mice (13). Normally, the life-time of a laboratory mouse is 1-2 years. The long-lived plasma cells may, therefore, play a far more important role in the life-long immunological memory in mice than was previous believed.

The down-regulation of many of the characteristic B cell markers during plasma cell differentiation, makes the detection of plasma cells by ordinary immunohistochemical staining more difficult. One molecule that is often referred to as a plasma cell marker is CD138 (Syndecan-1), but since it is also expressed on pre-B cells (14) it could be difficult to use it to detect plasma cells exclusively.

T CELLS

All T lymphocytes, or T cells, are characterised by the expression of the T cell receptor (TCR). On the cell surface, the TCR molecules are associated with CD3, a complex of polypeptides involved in intracellular signalling (15). The TCRs show a high degree of diversity, similar to that of the immunoglobulins. As with the immunoglobulins, the TCR-diversity is also generated by a procedure of randomly joining different gene segments together. Unlike the membrane-bound Ig, TCR does not recognise native
antigens, but interacts specifically with short, processed antigens in association with MHC molecules on cells (16). There are two main types of TCRs; TCR-1 and TCR-2. TCR-1 consists of disulphide-linked γ and δ polypeptides. TCR-2 is structurally similar, but is a heterodimer of α and β polypeptides. The TCR-1+ T cells constitute a minor subpopulation of circulating T cells and will, therefore, not be discussed further in this thesis.

TCR-2+ T cells can be subdivided into two distinct populations (15, 17). One subset carries the CD4 marker and mainly induces immune responses, whereas the other subset carries the CD8 marker and is predominantly cytotoxic.

**CD4+ T cells**

The function of CD4+ T cells is to “help” or “induce” immune responses and because of this, the cells are also called T-helper (Th) cells. Th cells have a central organising role in cell-mediated immunity and amongst other things, they fine-tune the specificity of the response, aid proliferation of the appropriate effector cells and enhance the function of phagocytes. The TCRs of Th cells bind to antigenic peptides associated with MHC class II molecules on antigen presenting cells. In order to activate the Th cell, additional interactions between molecules on the T cell and the antigen presenting cell are required. Once the Th cell is active, it will release several cytokines, which will then affect the immune response in different ways.

The Th cell population can be further divided into Th1, Th2 and Th0 cells, depending on which cytokines the cell releases. Th1 cells predominantly release IFNγ and IL-2, which will tend to activate macrophages for example. Th2 cells, on the other hand, release IL-4, IL-5, IL-6 and IL-10, which tend to increase production of eosinophils and mast cells and enhance antibody production, including IgE. Once established, each of these patterns of response can suppress the other, via the cytokines that are released (18). The Th0 cells are more intermediate in terms of their cytokine-production profile.

**CD8+ T cells**

The cytotoxic CD8+ T cells (Tc) act by destroying cells that they recognise as foreign. In many cases, the target cells are infected with viruses or other intracellular pathogens. The TCRs of Tc cells bind to antigens associated with MHC class I molecules. As with to the Th cells, the Tc cells need the
binding of the TCR to MHC molecules (in the case of Tc, class I) associated with antigenic peptide in order to lyse the target cell, as well as other interactions between different molecules. After lysis of the target cell the Tc cell survives and can continue to kill other target cells.

**T cell development**

The thymus is essential for the development and "education" of T cells. Stem cells from the foetal liver and later from the bone marrow, migrate into the thymus as a response to chemotactic signals. These stem cells will divide into a large number of immature T cells, known as thymocytes. During this maturation procedure, the thymocytes will undergo rearrangements of the TCR genes in order to obtain a high diversity of displayed TCRs.

Since T cells only recognise antigens associated with self-MHC molecules, a variety of APCs can be found in the thymus. To eliminate non-functional and autoreactive T cells, these APCs present antigens to the developing T cells. As a result, T cells expressing TCRs with no affinity, or very high affinity for MHC molecules will undergo apoptosis. T cells with intermediate affinity for MHC survive and continue along their pathway of maturation.

Not all self-reactive T cells are eliminated during intrathymic development and a separate mechanism is therefore required to prevent these surviving cells from attacking the body. If a peripheral T cell encounters and reacts to an extrathymic autoantigen it will become anergic.

**ANTIBODIES**

Antibodies, or immunoglobulins, are glycoproteins present in serum and tissue fluids of all mammals. Some of them act as B cell surface receptors, while others are secreted into the blood by plasma cells.

The antibody molecule consists of four protein chains; two light chains and two heavy chains, linked together by disulfide bonds. This four-chain molecule is known as the basic immunoglobulin structure, with the chains bound to each other in a "Y"-shaped configuration. Each immunoglobulin molecule is bifunctional, since one part of the antibody binds antigen and another is involved in mediating so-called effector functions. The two arms of the "Y"-structure are the variable regions that recognise and bind antigens. They are called the Fab fragment of the
immunoglobulin. The foot of the "Y" constitutes the part of the antibody that is constant within a class of immunoglobulins and is referred to as the Fc fragment. The Fc fragment can bind to Fc receptors on cells of the immune system and thereby exert different regulatory effects on the immune response.

Immunoglobulins can be divided into five distinct classes, namely IgG, IgM, IgA, IgD and IgE, which differ in size, charge, amino acid composition and carbohydrate content. The different classes of immunoglobulins also have different sets of functions, but only IgG and IgA will be further discussed here, since both are present in milk, although only IgG appears to be transported from the mother to the offspring.

**IgG**

IgG is the major immunoglobulin in normal serum of both humans and mice. It consists of a single four-chain molecule and is the major antibody of secondary immune responses. As will be further discussed later on, maternal IgG confers immunity in neonates. It is transported across the placenta in humans and across the intestinal epithelium in other mammals, such as mouse and pig. There are four subclasses of IgG, namely IgG1, IgG2a, IgG2b and IgG3.

IgG has been reported to suppress the immune response against large particulate antigens, such as erythrocytes (19-22), whereas IgG induces enhancement of the immune response in complex with soluble protein (19, 22). A more recent study, however, reports that the observed suppression of the immune response is not a “true” suppression, but rather the result of the blocking of antigenic determinants (23).

**IgA**

In most mammals, IgA occurs in a dimeric form of the four-chain structure. IgA is the predominant antibody in saliva, colostrum, milk and tracheobronchial and genito-urinary secretions. In order to join the subunits of dimeric IgA, a peptide chain called the J chain is needed. In addition, secretory IgA is always associated with a protein called the secretory piece, which facilitates the transport across membranes and protects the dimer from proteolytic cleavage.
MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULES

The question of how the immune system can distinguish “self” from “non-self” has fascinated scientists during the 20th century and many transplantation experiments have been performed throughout the years. One of the breakthroughs in transplantation immunology was the characterisation of the transplantation antigens. It was discovered that these molecules were of great importance for the acceptance or rejection of grafted tissue in mice (24). This ability of self/non-self discrimination was later mapped to what would be known as the major histocompatibility complex (MHC) region on chromosome 17 in mice (chromosome 6 in humans). The transplantation antigens were later shown to be highly polymorphic and were grouped into two classes with respect to both function and cell-distribution, namely MHC class I and II molecules. The human MHC molecules are also referred to as human leukocyte antigens (HLAs).

MHC class I

The MHC class I molecules are expressed on the surface of most nucleated cells in the body and present intracellular peptide antigens, such as viral peptides, to CD8+ T cells (25). The classical polymorphic MHC class I molecules are encoded by the genes H2-K, H2-D and H-2L in mice and HLA-A, HLA-B and HLA-C in humans. The class I molecule is a heterodimer of an α chain non-covalently associated with a β2-microglobulin polypeptide. The non-classical class I molecules in humans are encoded by the genes HLA-E, HLA-F and HLA-G. In contrast to the classical class I molecules, the non-classical ones show a restricted tissue distribution, low degree of polymorphism and are expressed in lower amounts (26).

MHC class II

In contrast to the MHC class I molecules, MHC class II molecules are only expressed on the surface of cells of the immune system and on antigen presenting cells. Other cell types can, however, be induced to express MHC class II by exposure to, for example, IFNγ or IL-4 (reviewed in (27)). The products of the classical MHC class II genes (H2-A and H2-E in the mouse; HLA-DR, HLA-DQ and HLA-DP in humans) are heterodimers comprised of α and β glycoprotein chains. Each chain has five domains, of which the outermost, α1 and β1, together form the peptide-binding groove.
There are also non-classical MHC class II genes, which are non-polymorphic and encode molecules such as H2-M (HLA-DM in humans) (28, 29) and H2-O (HLA-DO in humans) (30, 31).

**Regulation of MHC class II gene expression**

The expression of MHC class II genes can be either constitutive or cytokine-induced. Several different signals, of which IFNγ is the most potent, induce class II expression in a large variety of cells (reviewed in (32)). The constitutive expression is, as previously mentioned, mainly limited to antigen presenting cells. Both modes are controlled primarily at the level of transcription, via highly conserved cis-acting sequences, called the W, X and Y boxes, which are all located upstream of the start site of transcription (reviewed in (33, 34)). Several distinct protein complexes are known to interact with the X1, X2 and Y motifs, thereby affecting transcription (reviewed in (33, 34)).

Bare lymphocyte syndrome (BLS) is a hereditary disease characterised by a severe immunodeficiency, which is caused by MHC class II-deficiency (35, 36). Both the constitutive and inducible expression is affected and it is known that the defects responsible for the disease reside not in the class II genes themselves, but in trans-acting regulatory factors that are essential for their expression (35). Studies on B cell lines from BLS patients have led to the identification of three factors that can restore MHC class II expression in these cell lines, namely regulatory factor X (RFX) 5 (37), CIITA (class II transactivator) (38) and RFX-AP (39). RFX5 and RFXAP are components of the RFX-complex, which is known to bind specifically to the X box. The presence of RFX is necessary for promoter activity, but its presence is not sufficient to activate gene expression.

CIITA is essential for both constitutive and IFNγ-inducible expression of MHC class II genes (38, 40, 41) and plays a key role in the extinction of MHC class II gene expression in plasma cells (42). It does not interact directly with DNA (38, 43, 44) and is induced by IFNγ in a time frame that precedes MHC class II gene expression (40,41,45). The importance of the role of CIITA in MHC class II expression was further demonstrated in CIITA-deficient mice (46). Class II expression, both constitutive and inducible, was lacking in these mice, except for low expression in a subset of thymic epithelial cells. In addition to the effect on the expression of the classical MHC class II genes, CIITA regulates the expression of the non-classical MHC class II molecule HLA-DM (H2-M in mice) and the invariant chain (47, 48). These molecules
are of great importance for peptide loading and antigen presentation via MHC class II, as is further outlined below.

**Peptide loading and antigen presentation via MHC class II**

Antigen presentation via MHC class II molecules is a central feature in an immune response against foreign invaders. The entire procedure, from peptide processing to the transport of the MHC class II-peptide complex to the cell surface, is very complicated and involves many different steps and components. Several “professional” antigen presenting cells (APCs), such as dendritic cells, B cells and macrophages, as well as non-professional APCs such as activated epithelial cells can internalise antigens and then display them on MHC class II molecules at the cell surface for recognition by CD4+ Th cells. The interaction between the APC and the Th cell involves not only the MHC class II molecules and TCRs, but also several other surface molecules, resulting in an activation of the T cell and a release of a number of immunomodulatory cytokines that can, for instance, activate the APC. In contrast to MHC class I molecules, which are loaded with peptides in the endoplasmic reticulum (ER), MHC class II molecules are loaded in endosomal/lysosomal compartments with peptides that are derived from endocytosed proteins (49).

Like other transmembrane proteins, MHC class II molecules are translocated into the ER after synthesis, where they associate with another protein called the invariant chain (Ii). The Ii directs the membrane trafficking of the αβ dimer (50, 51) and prevents class II molecules from binding peptides in the ER and during the transit through the secretory pathway (52-54). Sorting signals in the cytoplasmic tail and transmembrane region of the Ii guide the αβ-Ii complex to endosomal/lysosomal compartments (55), where the Ii is proteolytically degraded. Short peptides from Ii, called CLIP (class II associated invariant chain peptides), are not degraded and one of these stays in the binding-cleft of the class II molecule (56). As long as CLIP is bound to the groove, the MHC class II molecule cannot be loaded with other peptides. To facilitate the release of CLIP from the groove and promote the loading of antigenic peptides, a non-classical MHC class II molecule called HLA-DM (the murine equivalent is called H2-M) is needed (reviewed in (57). Recently, yet another member of the non-classical MHC class II family, called HLA-DO (H2-O in mice), was characterised (31). HLA-DO is found in complex with HLA-DM, but its exact function is debated (reviewed in (58)). Eventually, the MHC class II molecule
loaded with an antigenic peptide is transported to the cell surface, where the peptide is presented to CD4+ Th cells.

**Fc RECEPTORS**

The Fc portion of antibodies interacts with the cells of the immune system via receptors called Fc receptors (FcR), which are present on the surface of phagocytic cells and some lymphocytes. This means that an antibody, that has bound an antigen, can attach to a phagocytic cell and thereby ensure that the phagocytic cell will engulf the antigen.

The FcRs are also important in mediating signalling from the cell-surface into the cell-interior. To deliver such signals to cells, FcRs need to be aggregated at the cell surface by antibodies or multivalent antigens. It has been shown in several studies, that FcR-aggregation, rather than ligand binding, is crucial for the generation of a signal (59-61). There are Fc receptors for every antibody class; FcγR binds IgG, FcεR binds IgE, FcαR binds IgA, FcµR binds IgM and FcδR binds IgD. Most human and murine FcRs are members of the immunoglobulin superfamily; others belong to lectin families. In this thesis, only the FcγR will be further described.

The “classical” Fcγ receptors have two or three immunoglobulin-like extracellular domains that bind the Fc portion of IgG. The receptors can either be constituted by one single chain, or by several chains. Single-chain FcγR are low-affinity receptors, while multi-chain receptors show both high, intermediate and low affinity for IgG. There are three major types of “classical” Fcγ receptors identified; FcγRI, FcγRII and FcγRIII. FcγRI (CD64) is a high affinity receptor expressed primarily on macrophages, while FcγRIII (CD16) is a low affinity receptor found on mononuclear phagocytes, granulocytes, platelets and NK cells. FcγRII (CD32) is a heterogenous, low affinity receptor, which is present on B cells, mononuclear phagocytes, granulocytes and platelets.

Crosslinking of FcγRII and membrane-Ig (mIg) on B cells strongly inhibits activation and differentiation of the B cells (62, 63). The crosslinking inhibits the signalling via the mlg, through an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic region of FcγRIIb1 (reviewed in (64)). FcγRIIB-deficient mice have been shown to have augmented humoral responses, thereby indicating an in vivo immunoregulatory role for FcγRIIB (65).
The neonatal Fc receptor

The Fc receptor, which mediates the transfer of maternal IgG over the intestinal epithelium in neonates was cloned and characterised by Simister and colleagues (66, 67). It was called FcRn, since it was first found in newborn rats. In analogy to MHC class I molecules, FcRn consists of β2-microglobulin, associated with an integral membrane α-chain, which makes it different from the "classical" FcγR. This MHC class I-like receptor has also been found on rat hepatocytes (68), in foetal yolk sac of rats (69) and mice (70), on syncytiotrophoblasts of the human placenta (71, 72) and on human intestinal cells (73).

The binding of IgG to FcRn is pH dependent. If the pH is increased from pH 6 to pH 7, the affinity constant is decreased by two orders of magnitude (74), which means that IgG can bind to the receptor in the acidic environment at the apical part of the neonatal intestinal cells.

It has also been reported that β2-microglobulin-deficient mice, which lack FcRn, have a marked acceleration of the clearance of serum-IgG as compared to wild-type mice (75) and that FcRn could, therefore, play an important role for the half-life of IgG in normal mice (reviewed in(76)).

FROM FERTILISATION TO PLACENTATION

Mice normally mate at night, when the female is in oestrus. The oocytes are released from the ovariun into the ampulla of the oviduct, where fertilisation takes place. The zygotes slowly start dividing and move through the oviduct to the uterus. At about gestation day 3.5-4, the embryos, which now are at the blastocyst stage, reach the uterus, where they have to hatch from their zona pellucidae before the process of implantation can be initiated. At the time of implantation, the blastocyst aligns with the uterine wall and the trophectoderm cells attach to the uterine epithelium, which is only competent to receive the embryo during a short period about four days after copulation. The trophectoderm, with its invasive capacity, will start the erosion of the maternal uterine epithelium, invade the uterine mucosa and finally, contribute to the formation of the placenta and the foetal membranes. The inner cell mass of the blastocyst will form the embryo proper.

The implantation of the blastocyst induces the endometrial stroma to thicken and become highly vascularised. This gives rise to the decidua, which will constitute the maternal part of the definitive placenta. In the rodent placenta, the area of materno-foetal interdigitation is constituted by a
Figure 1. Schematic picture of a murine foetus and placenta at mid-gestation.

complex framework of maternal and foetal tissues. This area, the labyrinthine trophoblast, is formed as allantoic blood vessels invade the part of the ectoplacental cone closest to the embryo. The spongiotrophoblast (basal zone in rats) is formed by the part of the ectoplacental cone that is not invaded by the allantoic blood vessels. Giant trophoblast cells constitute the borderline to the maternal decidua. The maternal and foetal circulations are normally kept separate during the entire course of gestation. The placental membrane separating the circulations consists of three layers of trophoblast cells (77). This means that the trophoblast cells are flooded with maternal blood, including the white blood cells and proteins that it contains.

The placenta will transport nutrients from the mother to the developing foetus, as well as foetal waste products in the opposite direction. Furthermore, the placenta serves as an important endocrine organ secreting female hormones and growth factors that play a vital role in maintaining pregnancy and supporting foetal growth (78).
THE IMMUNOLOGICAL PARADOX OF PREGNANCY

During mammalian pregnancy, the foetus, which expresses foreign (paternal) antigens, is carried within the maternal body and is in close contact with maternal blood. This intricate relationship requires very special immunomodulatory actions, since the foetus has to be protected from maternal immunological attacks and rejection, while at the same time, the maternal immune system has to ensure protection against infectious agents for both the mother and the foetus. This enigma is sometimes referred to as the "immunological paradox of pregnancy" and throughout the years, many scientists have tried to find the immunological explanation as to why pregnancy can succeed at all, during these conditions.

As mentioned above, the foetus expresses both maternal and paternal MHC molecules, which means that it can be considered as a form of allograft from an immunological point of view. Numerous investigations aimed at finding the key to nature’s successful transplantation have been performed during the last century and as early as 1953, Medawar summarised a number of theories that raised possible explanations for how the foetus is protected from immunological rejection (79). Although many of these theories were later on refuted, they gave rise to a wave of research in this scientific area. Today it is clear that the placenta, with its special properties, is of central importance for the survival of the foetal allograft. The exact mechanisms by which the foetus avoids immunological recognition and rejection have still not been entirely defined, but several have been suggested to contribute to the non-rejection of the foetus. Some of these will be further outlined below.

Placental expression of MHC class I

MHC molecules are known to be of central importance for the rejection or acceptance of grafted tissues and organs. Consequently, the placental expression patterns of these molecules have been the foci of many investigations aimed at solving the immunological mystery of foetal survival.

Although the expression pattern of MHC in the placenta is very restricted, MHC class I expression has been reported in the spongiotrophoblast region of mice and in the corresponding basal zone in rats (reviewed in (80)). In the human placenta, various expression patterns of HLA class I have been detected (reviewed in (81, 82)). HLA-A and -B are normally absent, while HLA-C has been detected in extravillous cytotrophoblast (83).
The non-polymorphic HLA-G is strongly expressed in the extravillous cytotrophoblast (84, 85). The placental function of HLA-G is not entirely defined, but it has been suggested to protect the foetal cells from cell-lysis by maternal NK cells (86). In accordance with this idea, several in vitro studies have shown that HLA-G-transfected cells are protected from NK cell-mediated cell-lysis (87-89). A recent study reported that HLA-G may also inhibit NK cell migration across endothelial cells in the placenta (90). To date, no murine homologue to HLA-G has been identified.

Recently, it was reported that another non-classical HLA class I molecule with limited polymorphism, namely HLA-E, is expressed on trophoblast cells and that it interacts with decidual NK cells in vitro (91).

Classical MHC class I molecules have not been detected in the area of maternal-foetal exchange in either humans (the syncytiotrophoblast) or mice (the labyrinthine trophoblast). Interestingly, it has been reported that expression of allogeneic MHC class I molecules in both the spongiotrophoblast and the labyrinthine trophoblast of transgenic mice do not interfere with the normal course of pregnancy (92). Direct evidence for placental cell-surface expression of the transgenic MHC molecules was, however, not presented (92), which leaves the possibility that the class I molecules were retained in the cytoplasm, as has been reported to be the case for paternally-derived class I molecules in the rat placenta (93).

**Placental absence of MHC class II**

MHC class II molecules, on the other hand, are not expressed at all in the human or mouse placenta (94-96). The total absence of MHC class II expression has been suggested to be of importance for the avoidance of maternal immune rejection (79) and several attempts to induce placental expression of class II in mice have been reported. It was shown that in vivo treatment of pregnant mice with IFNγ fail to induce placental class II expression in mice (97, 98). Other investigators, however, published contradictory results (99, 100), stating that treatment of pregnant mice with IFNγ did indeed induce class II expression in the spongiotrophoblast region and that this lead to foetal abortion due to maternal immune reactions (100). Similarly, treatment of pregnant mice with the methylation inhibitor 5-azacytidine was reported to induce MHC class II expression in the labyrinthine region, with subsequent foetal rejection (101). The question of whether it is possible to induce endogenous placental expression of MHC class II, therefore, requires further investigation.
Placental expression of Fas ligand

Another mechanism that has been suggested to contribute to the non-rejection of the trophoblast is the expression of Fas ligand (FasL). The cell-surface molecules Fas (CD95) and FasL are members of the TNF/NGF-receptor superfamily and their interaction leads to apoptosis of cells expressing Fas (102). Fas is expressed on many different types of cells, while FasL expression is restricted to immune-privileged tissues such as cells in the cornea, Sertoli cells in the testis and cells of the immune system itself (103). In mice, Fas L is also expressed on uterine glandular epithelial cells, decidual cells and placental trophoblast cells. The predominant expression pattern shifts through pregnancy from uterus to placenta (104). The Fas/FasL interaction is important in the elimination of self-reactive T cells and consequently, the placental expression of FasL would confer immune-privilege by inducing apoptosis in maternal Fas+ T cells. The importance of placental FasL expression for the success of pregnancy is demonstrated in mice lacking functional FasL expression (gld mice). These mice show extensive leukocyte infiltrates at the placental-decidual interface and have a higher frequency of foetal resorptions (104). A mandatory role for the Fas/FasL system in allogeneic pregnancy, however, has recently been disputed (105).

Placental expression of complement regulatory proteins

Yet another protective mechanism suggested to be used by the trophoblast is the expression of several complement regulatory proteins. From the 6th week of gestation in humans, the trophoblast has been shown to express CD46 (membrane co-factor protein) and CD55 (decay accelerating factor), which inhibit C3 convertase activity, as well as CD59, which prevents membrane attack complex formation at the terminal stage of complement activation (106). Recently, the in vivo role of a murine complement regulatory protein with CD46- and CD55-like activity has been investigated (107). This protein is encoded by the gene Crry 9, which is normally strongly expressed in the placenta. Disruption of this gene caused complement deposition and placental inflammation in pregnant mice.
Placental tryptophan metabolism

In 1998 it was reported that the placental expression of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) prevents rejection of allogeneic foetuses in mice (108). The level of tryptophan at the maternal-foetal interface is very low, partly because of the transplacental transport of tryptophan and partly due to the IDO-dependent consumption of residual tryptophan. T cells appear to be very sensitive to the levels of free tryptophan and on the basis of this, Munn and co-workers suggested that IDO suppresses T cell activation and proliferation in local microenvironments by "nutrient depletion" or "starvation" (108). One observation that may support their hypothesis, is that immunosuppressive human macrophages have been reported to prevent T cell-activation in vitro by depriving T cells of tryptophan (109). The exact mechanism by which IDO contributes to the protection of the allogeneic foetus, however, has to be further elucidated.

Alteration in cytokine-balance during pregnancy

A large number of different cytokines are produced by uterine, decidual and trophoblast cells (110). These include colony-stimulating factors, IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, type 1 interferon, IFNγ, TNFα and TGFβ. The exact role of the different expression patterns of these cytokines during different gestational stages is not entirely clear, but the general opinion is that an appropriate cytokine-balance at the maternal-foetal interface is necessary for successful pregnancy.

It has been suggested that there is a switch to, or bias for, Th2-responses during pregnancy. As mentioned before, a Th2-response is characterised by the production of Th2-specific cytokines such as IL-4 and IL-10. These cytokines will induce humoral immune responses to a larger extent and inhibit the production of Th1-cytokines, which are associated with cell-mediated immunity.

The suggestion of a Th2-bias during pregnancy is primarily based on several observations in mice. It has been shown that injections of the Th1-cytokine IL-2 during early murine pregnancy increase the rates of foetal loss (111). In agreement with this, gestational tissues harvested from mice undergoing spontaneous pregnancy loss have been reported to show a bias for Th1-cytokine production (112). It has also been reported that IL-10 prevents foetal wastage in an abortion-prone crossing of mice, in which the placental levels of IL-10 are low (113). Taken together, these observations
indicate that IL-10 and/or other Th2-cytokines may play an important role in preventing foetal rejection.

TRANSMISSION OF PASSIVE IMMUNITY

Not only should the offspring be protected from maternal immunological rejection during gestation, but it should also be protected from infections, via transmission of passive immunity from the mother.

As early as 1892, Paul Erlich performed the first critical experiments in paedriatric immunology, demonstrating the manner in which the foetus and neonate acquire protective immunity from the mother and the importance of milk antibodies. Using elegant experimental mouse models, he was able to show that immunity to plant toxins was transferred from immune mothers to their foetuses in utero, as well as postnatally via the milk (114).

The area of maternal transmission of passive immunity has been extensively studied during the 20th century. It has been shown that this phenomenon occurs in most mammalian species and that it involves a specific transport of IgG from mother to offspring. The route of and time point for the antibody-transport vary among different species (reviewed in (115)). For instance, humans and guinea pigs receive most of the maternal IgG via the placenta before birth, while cattle and horses only receive maternal IgG from colostrum. There are also species, such as mice and rats, in which some prenatal antibody-transport occurs, although most of the maternal antibodies are acquired postnatally from the milk and colostrum.

Antibody transmission via the placenta

For the immunological defence of the human infant, active transport of maternal IgG across the placenta is very important. The foetal synthesis of IgG is very low (116) and most of the IgG in newborns is of maternal origin (117). In order to reach the foetal circulation, the IgG antibodies have to be transported across to two cellular barries; the syncytiotrophoblast, which is the outermost layer of the chorionic villi and is in contact with maternal blood; and the foetal capillary endothelium.

IgG and several IgG-binding proteins have been detected in the syncytiotrophoblast (118-120). Recently, expression of the human homologue of the neonatal Fc receptor, hFcRn, was also found in the syncytiotrophoblast of the placenta (71, 72). As has been proposed by several
investigators (71, 72, 121), this receptor is the most likely candidate responsible for the transport of maternal antibodies across the placenta. Since hFcRn, as with rodent FcRn, binds IgG at pH 6 but not at pH 7.5 (121), maternal IgG cannot be "loaded" on the receptor in contact with maternal blood. The binding of IgG could, however, occur in acidic endosomes with a pH near to 6. This view is supported by the fact that the hFcRn has been detected in cytoplasmic granules and not on syncytiotrophoblast plasma membranes (122). The way in which IgG is delivered into endosomes, as well as the mechanism of IgG transport across the foetal capillary endothelium, still remain unclear.

As previously mentioned, mice and rats receive relatively small amounts of maternal IgG via the placenta. The transport of IgG from the maternal circulation begins between the eleventh and twelfth days of gestation in mice (123). In rats, maternal IgG can be detected after 17 days of gestational age (115). Routes of transmission exist across the yolk sac splanchnopleure, placenta and foetal intestine, but their relative importance is not known (115).

**Antibody transmission via the intestinal route**

Rats and mice acquire maternal IgG by the suckling of milk, from birth until the day of weaning (115). As previously mentioned, FcRn is the only IgG transporter in the neonatal gut of rodents. Since the pH in the neonatal intestine is around 6, IgG can bind to the FcRn at the luminal part of the cell. The acidic pH is not needed, however, for IgG transport across segments of neonatal rat proximal small intestine *in vitro* (124), suggesting that IgG might bind to the receptor after uptake in acidified endosomes. The mechanism of transport might, therefore, be similar to the mechanism suggested for placental transport of IgG.

In mice, the absorption of IgG ceases around day 16 of age. This time point is known as the time of "gut closure". The phenomenon depends on the fact that the neonatal intestinal epithelial cells differentiate into mature cells and thereby lose their ability to take up macromolecules (reviewed in (125)).

Recently, the hFcRn has been found on human intestinal epithelial cells (73) and the authors suggest that potential roles for hFcRn in the intestine could include the transfer of passive immunity, induction of oral tolerance and immuno-surveillance.
Protective role of IgA

The milk of humans and mice contains high amounts of not only IgG, but also IgA (126, 127), which is present in a form known as secretory IgA (sIgA). The sIgA is resistant to pH changes and proteolytic enzymes and can, therefore, largely withstand the digestive processes in the alimentary tract of the neonate. It is believed that sIgA exerts its protective role in the gut lumen by binding to microbes, thereby blocking their adherence and penetration of the gut epithelium. In contrast to IgG, sIgA is not believed to be taken up into the neonatal circulation.

MATERNAL-FOETAL EXCHANGE OF CELLS

Although the placenta blocks the passage of many micro-organisms and macro-molecules, maternal as well as foetal cells occasionally cross the barrier. This type of transplacental transfer of cells between the mother and the foetus could perhaps be explained by small-scale haemorrhages within the placenta, that would give rise to the leakage of cells, although other types of accidental cell-transfer cannot be excluded. Foetal trophoblast cells have been detected in uterine venous blood, lung parenchyma and peripheral blood of pregnant women (128-130).

Transfer of cells in the opposite direction, i.e. from the mother to the foetus, has also been reported. Maternal T cells have been detected in immunodeficient patients (131) and a maternal NK-cell-lymphoma has been demonstrated to invade the foetus (132).

Although the murine placenta is believed to be more impermeable to cells than the human placenta, maternal cells have been detected in mouse foetuses (133-135). It has also been reported that maternal green fluorescent protein (GFP)-positive cells were transported from the GFP-transgenic mother to the GFP-negative foetuses (136). The maternal cells showed a heterogenous distribution between different organs and tissues in the foetuses (135, 136). The effects on foetal survival was, however, not elucidated.

MATERNAL LEUKOCYTES IN MILK

It is well known that milk and colostrum from most mammalian species, including humans, contain significant numbers of viable maternal cells. In the beginning of the 20th century, it was believed that presence of large
numbers of cells in milk indicated pathological conditions in the udder (137) and as a consequence, the cell content was used as an estimate of the sanitary state of the milk. Today, however, there is no doubt that bovine milk, from healthy animals, normally contains considerable numbers of cells.

Apart from a small proportion of epithelial cells, leukocytes such as macrophages, B and T cells, constitute the majority of the cells present in milk. The leukocyte concentration of milk has been estimated to be about $1.5 \times 10^6$ cells/ml (138).

The significance of milk leukocytes is not fully understood, although they are suggested to protect the gut lumen of the neonate. In addition, it has been shown that milk cells can traverse the neonatal intestinal epithelium in several species, including pigs (139), sheep (140, 141), rats (140) and mice(142). The transfer of maternal milk cells in mice, however, is disputed in other studies (143-145).
AIMS OF THE PRESENT INVESTIGATION

The papers included in this thesis, deal with different aspects of immunological interactions between the mother and her offspring in mice and can be divided into two main lines of research. The general aim of the first part of the thesis (paper I, II and III) was to investigate some of the mechanisms involved in the foetal avoidance of maternal rejection reactions. The second part of the thesis (paper IV, V and VI) was aimed at studying transmission of passive immunity from the mother to the offspring and different effects of such a transfer on the immune system and the immune response, of both immunodeficient and normal offspring. The specific aims are outlined below.

- Is it possible to induce MHC class II expression on cells of trophoblast origin in vivo or in culture?
- Are the Th2 cytokines IL-4 and IL-10 crucial for the completion of murine allogeneic pregnancy?
- Can functional B-lineage or Ig-secreting cells of maternal origin be transferred to the offspring?
- What are the effects of neonatal ingestion of Ig-containing milk on the outcome of adult B cell-transfer to non-irradiated B cell-deficient recipients?
- How does lack of maternal antibody transmission affect the immune system and the immune responses of normal offspring?
TRANSGENIC ANIMAL MODELS

In the studies included in this thesis (except for paper II), different transgenic mouse strains have been used as model animals and tools in the experiments. The strains and animal models of central importance will be described here, while other models will only be described in the original papers themselves.

IL-4- AND IL-10-DEFICIENT MICE (used in paper III)

Mice homozygous for a mutation that inactivates the IL-4 gene were generated by Kühn et al (146). The serum levels of IgG1 and IgE are strongly reduced in these mice, although the development of B cells and T cells appears normal (146). The mice look surprisingly healthy, however, and under specified pathogen-free (SPF) conditions, the IL-4 deficient mice are relatively easily bred and no obvious reproductive failure can be observed.

The IL-10 deficient mice were produced by the same group as the IL-4 deficient mice (147). In these mice, the lymphocyte development and antibody responses are normal, but most animals are growth retarded, anaemic and suffer from chronic enterocolitis (147). The onset of the disease varies among the animals and in younger animals, it is often less severe. Other factors probably influencing the onset and severity of the disease are the genetic background of the animals and the pathogenic pressure in the environment (147). Animals bred in a SPF environment often suffer from local colitis, rather than the general enterocolitis, that is observed in mutants from conventional environments.

The IL-10 deficient mice do indeed have problems in reproductive success, although it should be kept in mind that the symptoms of severe enterocolitis could interfere with reproduction. It is possible, however, to keep these mice in homozygous breeding in an SPF environment and preferentially use young animals for breeding.

In paper III, IL-4 deficient and IL-10 deficient mice were cross-bred to generate double deficient mice. The double deficient mice were used for syngeneic and allogeneic matings, as well as in embryo transfer experiments, to investigate the importance of the Th2-cytokines IL-4 and IL-10 for the completion of normal pregnancies.
B CELL-DEFICIENT MICE (used in paper IV, V, VI)

The B cell-deficient mice were originally produced by Kitamura and co-workers, by inserting a neomycin resistance gene in the transmembrane exon of the Ig µ chain (9). Due to this targeted mutation, the B cells of a B cell-deficient mouse cannot express surface IgM and are therefore arrested at the pre-B cell stage of development (148, 149). This means that the mouse lacks mature, functional B cells and thereby loses the ability to produce and secrete antibodies. This inability to secrete immunoglobulins, makes B cell-deficient mice useful for, amongst other things, studies on the role of endogenous Ig-secretion for various immunological and physiological functions and for studies of the fate of adoptively transferred B cells.

In conventional animal facilities, homozygous breeding of B cell-deficient mice is difficult, since many of them succumb to wasting syndrome (150). In a cleaner SPF-environment (without mouse hepatitis virus) the survival frequency of the B cell-deficient pups from homozygous breeding is, however, much higher (151).

GREEN FLUORESCENT PROTEIN-TRANSGENIC MICE (used in paper IV)

In the bioluminescent jellyfish *Aequorea victoria*, the green fluorescent protein (GFP) is the source of natural green fluorescence that can be observed in the dark. There are different mutants of GFP and the transgenic mice used in paper IV were produced by Okabe and co-workers and carry an enhanced green fluorescent protein (EGFP) cDNA under the control of a chicken beta-actin promoter and a cytomegalovirus enhancer (152). The GFP-transgenic mice are uniformly green when exposed to UV light, with the exception of hair and erythrocytes. The EGFP used was designed to be expressed and distributed throughout the cytosol (152). Unfortunately, this lead to some complications when trying to examine fresh cryosections of spleens from the B cell-deficient pups born to GFP-transgenic females (study IV) with the confocal laser microscope. As the cells were sectioned, the leakage of GFP made it even more difficult to find the low number of engrafted maternal cells *in situ*. This is the reason for examining spleen cell suspension, rather than cryosectioned spleens in the confocal microscope (study IV).
RESULTS AND DISCUSSION

IN VIVO TREATMENT WITH IFN\(_\gamma\) OR 5-AZACYTIDINE DOES NOT INDUCE PLACENTAL MHC CLASS II EXPRESSION IN MICE

Several attempts to induce endogenous placental MHC class II expression in mice have been reported, although with contradictory results. This study (Paper I) was performed to further clarify whether \textit{in vivo} treatment of pregnant mice with IFN\(_\gamma\) or the DNA methyltransferase inhibitor 5-azacytidine could induce placental MHC class II mRNA expression.

Pregnant mice were treated daily from gestation day (gd) 6.5 until gd 11.5, or from gd 11.5 until gd 17.5, with 5000 or 50000 IU IFNg or 10 µg 5-azacytidine. The effects of the treatments on placental class II induction were evaluated at gd 12.5 and 17.5, by \textit{in situ} hybridisation, ribonuclease protection assay (RPA) and immunohistochemistry.

Neither of the two IFN\(_\gamma\)-doses induced detectable placental MHC class II A\(β\)-chain mRNA expression at any of the time points analysed. The maternal decidua, however, showed induction of mRNA and protein expression at both gd 12.5 and gd 17.5. The early IFN\(_\gamma\)-treatment (gd 6.5-11) was not expected to induce placental class II expression, since the IFN\(_\gamma\)-receptor does not appear in the placenta until gd 12.5 (153). The early treatment was, therefore, only performed to mimic the protocol used by Vassiliadis \textit{et al} (100). During the later IFN\(_\gamma\)-treatment (gd 11.5-17.5), when IFN\(_\gamma\)-receptors are present, the observed lack of inducible MHC class II expression indicated that there might be a block in the signalling pathway.

None of the 5-azacytidine-treatments induced detectable placental class II mRNA expression, although a slight increase in mRNA expression was detected in the decidual and uterine tissues.

As previously mentioned, the class II transactivator (CIITA) is essential for both constitutive and inducible MHC class II gene expression. Recently, it was shown that CIITA gene expression is absent and not inducible by IFN\(_\gamma\) in trophoblast-derived cell lines (154, 155). Furthermore, the CIITA expression in trophoblast-derived cells appears to be prevented by a failure to recruit regulatory factors to the promoter (156). A considerable body of evidence suggests that methylation of CpG dinucleotides affects gene transcription (reviewed in (157)) and the CIITA silencing in trophoblast-derived cells is suggested to be due to methylation of CpGs within promoter pIV (156). The 5-azacytidine-treatment in paper I did not result in placental MHC class II expression. This could indicate that methylation of CIITA is not involved in the silencing of the class II genes \textit{in vivo}, but since the
methylation status of the CIITA gene was not examined either before or after the treatment, it is difficult to draw any conclusions as to whether demethylation of the CIITA gene was achieved. A similar study in rat trophoblast cells, however, showed that treatment with 5-azacytidine resulted in demethylation of the otherwise heavily methylated MHC class II promoter regions, but that this did not induce class II gene expression (158). The CIITA methylation status, however, was not examined.

TGF-β has been shown to suppress IFNγ-inducible MHC class II expression by inhibiting CIITA mRNA expression (159) and since the placenta produces considerable amounts of TGF-β, this could contribute to the absence of placental MHC class II in vivo.

Taken together, the results from paper I, however, strengthen the opinion that MHC class II expression cannot normally be induced in murine placental cells after IFNγ or 5-azacytidine treatments. The mechanisms by which placental class II expression is prohibited still remains unclear.

**ECTOPIC MHC CLASS II TRANSCRIPTION CAN RESULT IN SURFACE-EXPRESSION ON CELLS OF TROPHOBLAST ORIGIN**

In paper II, another approach was used to try to induce functional cell-surface expression of MHC class II molecules on cells of trophoblast origin. The murine cell line SM9-1, originating from a gd 9 placenta, as well as the human choriocarcinoma-derived cell line JEG-3 were transiently co-transfected with MHC class II Aq a and b genes under the control of strong viral promoter systems. The transfected cells were analysed for cell surface expression of class II molecules and assayed for antigen presentation ability in vitro.

Only a small proportion of the SM9-1 cells showed detectable surface expression of class II molecules. This appeared to be an effect of low transfection efficiency, as indicated by transfections of a β-galactosidase reporter constructs, and probably does not reflect the ability of the cells to express the class II genes. This did, however, make further functional studies of this cell line difficult.

A high proportion of the transfected JEG-3 cells showed distinct surface expression of murine MHC class II Aq molecules and in the antigen presentation assay, they efficiently activated the Aq-restricted T cell hybridoma. This indicated that the expressed α- and β-chains had been properly associated and attached to the cell-membrane. The fact that the T cell hybridoma was activated only when processed peptides were present and not in the presence of native unprocessed antigen, suggested that the
antigen was bound to the class II molecule at the cell surface and that the transfected cells could not function as antigen presenting cells.

It is well known that accessory proteins such as invariant chain (Ii) and H2-M (HLA-DM in humans) are necessary for efficient and functional antigen presentation by MHC class II molecules. Since the expression of Ii, H2-M and MHC class II is co-regulated in most instances (reviewed in (32)), it was not likely that the SM9-1 cells expressed these accessory molecules. H2-M is certainly absent in JEG-3 cells. Taken together, this suggests that the importance of accessory functions for the appearance of MHC class II molecules at the cell surface can be overcome by transient ectopic over-expression of the a and b genes, which could be expected to increase the random association of α- and β-chains. Whether such levels of over-expression can also be obtained in vivo using integrated transgenes is uncertain. The transgenic mice denoted CBQ, which were used as positive controls in paper I, carried the same MHC class II Aq β-chain gene construct as was used in the in vitro studies in paper II. Another transgenic mouse strain carrying the gene encoding class II Aq α-chain (denoted SAQ) that was used in paper II, was also generated. Because of very low expression levels of the SAQ transgene it was, however, difficult to obtain conclusive results. Such a transgenic mouse model could, otherwise, perhaps be one way to study the effects of placental MHC class II expression on foetal survival.

IL-4 AND IL-10 ARE NOT CRUCIAL FOR SUCCESSFUL PREGNANCY

The success of mammalian pregnancy has been suggested to partly be dependent on a Th2-cytokine bias at the maternal-foetal interface (160, 161). Foetal abortion in mice has been reported to be correlated with increased levels of Th1-cytokines (112) and low levels of IL-10 (113). Based on these and other observations, the Th2-cytokines IL-4 and IL-10 have been proposed to contribute to foetal survival by inhibiting Th1-responses at the maternal-foetal interface. The effect of a total lack of IL-4 and IL-10 on the outcome of pregnancy has not to my knowledge, however, been directly investigated.

To analyse the importance of a lack of either maternal or foetal IL-4 and IL-10 production on the outcome of allogeneic pregnancy, the reproductive performance of IL-4- and IL-10-double-deficient mice was studied in different mating combinations, as well as in embryo transfer experiments (Paper III).

The mating combination where the double deficient female was carrying H2-q and the wild type male carried H2-r (B10.Q x B10.R) made the mother and the foetuses differ by means of MHC only. All investigated
pregnancies resulting from this mating combination gave rise to pups and the litter sizes did not differ from the control group in which wild type B10.Q females were mated with B10.R males. Similarly, there was no difference in litter size between the groups in which female IL-4+/+IL-10⁻/⁻ or IL-4⁻/⁻IL-10⁻/⁻ B10.Q mice were mated with male BALB/c (H2-d) mice, as compared to the control group with female wild type B10.Q. Taken together, the results from these breeding experiments showed that even if mothers and foetuses are genetically very different, pregnancies remain unaffected by maternal lack of either IL-10 alone or IL-10 together with IL-4.

To investigate the importance of foetal production of IL-4 and IL-10 for foetal survival, IL-4⁻/⁻IL-10⁻/⁻ one-cell embryos (B10.Q carrying H2-q) were transferred to pseudopregnant foster mothers (C57BL/6 x CBA carrying H2-b and H2-k). As in the breeding experiment above, there was no difference in the number of delivered pups between the experimental group and the control group, in which the embryos were wild type B10.Q.

In summary, the data obtained from this study (paper III) showed that neither maternal nor foetal production of IL-4 and IL-10 were crucial for the completion of allogeneic pregnancies in mice, providing that the mice did not suffer from severe enterocolitis, which is a common complication in IL-10 deficient mice (147). If the role of IL-4 and IL-10 at the maternal-foetal interface is to inhibit the potentially harmful Th1-reactions, there seems to be some kind of redundancy in the system so that other mechanisms/cytokines still can ensure foetal survival.

MATERNAL Ig-SECRETING CELLS CAN INFILTRATE IMMUNODEFICIENT OFFSPRING

The reproductive performance of B cell-deficient (µ⁻/⁻) mice has previously been studied at our laboratory (150). During the breeding of the µ⁻/⁻ mice, it was observed that some of the mice showed clearly detectable levels of serum-IgG for prolonged periods of time when born to µ⁺/⁻ mothers. This study (paper IV) was initiated to investigate this phenomenon.

In order to evaluate whether a maternal transmission of functional B-lineage cells could occur, µ⁻/⁻ mice from heterozygous breeding (µ⁺/⁻ x µ⁻/⁻), as well as µ⁺/⁻ mice from homozygous breeding (µ⁻/⁻ x µ⁻/⁻) postnatally transferred to lactating µ⁺/+ foster dams, were analysed.

A proportion of the µ⁻/⁻ offspring born to µ⁺/⁻ mothers characteristically were serum-IgG positive up to 18 weeks of age, although B220⁺ or CD19⁺ B cells could not be detected in the spleen by flowcytometry. Interestingly, five out of nine mice displayed clearly detectable levels of IgG-
secreting cells in spleen and bone marrow (femur). This indicated that there could have been a transfer of maternal B-lineage cells to the offspring. The difficulty of detecting a splenic B220+ or CD19+ population in any of the μ⁻/⁻ mice that harboured Ig-secreting cells, favours the view that long-lived maternal plasma cells, rather than B cells are transmitted. The life-span of certain populations of plasma cells have been estimated to be more than one year in mice (13). It is also known that one single plasma cell can secrete 10 000 Ig molecules per second (11, 12), which means that even very small numbers of Ig-secreting cells could produce amounts of IgG that would be clearly detectable in an ELISA. This could explain why Ig-secreting cells were not detected in all the serum-IgG positive μ⁻/⁻ mice. It should also be mentioned that only a limited part of the lymphoid compartments in each

![Diagram of two possible routes of transmission of maternal Ig-secreting cells to the offspring: (1) Foetal uptake, (2) Neonatal uptake.](image)
mouse was screened for Ig-secreting cells. It is likely, therefore, that a higher proportion of the mice actually harbour maternal cells, but in other tissues than those analysed.

Seven out of ten \( \mu^{-/-} \) mice that originated from homozygous \( (\mu^{-/-} \times \mu^{-/-}) \) breeding, but had suckled a \( \mu^{+/+} \) foster mother were serum-IgG positive after three months. In two out of these seven, splenic IgG-secreting cells were detected. This suggested that maternal Ig-secreting cells could be transmitted postnatally via the milk.

To show that cells of maternal origin actually could be found in the offspring, \( \mu^{-/-} \text{GFP}^{-/-} \) mice born to \( \mu^{+/+} \text{GFP}^{+/+} \) mothers were analysed. In eight out of eleven \( \mu^{-/-} \text{GFP}^{-/-} \) offspring born to \( \mu^{+/+} \text{GFP}^{+/+} \) serum-IgG and/or Ig-secreting cells were detected. In three out of these eight mice, GFP\(^+\) cells were detected, showing that maternal cells had indeed infiltrated the offspring.

Although the actual mechanism(s) of transfer of maternal cells to the offspring, as well as the frequency and physiological relevance of such transfer in immuno-competent mice remains unclear, the results from this study could be of importance in, for example, developmental biology and immunology.

**NEONATAL SUCKLING OF Ig-CONTAINING MILK RESULTS IN INCREASED SURVIVAL OF B-LINEAGE CELL TRANSPLANTS**

Since B cell-deficient \( (\mu^{-/-}) \) mice lack endogenous mature B cells, they could be used as excellent model animals for investigating the fate of adoptively transferred B cells. Early studies report, however, that the majority of transferred B cells fail to become established in the lymphoid organs of mice (162, 163). In a more recent study using \( \mu^{-/-} \) mice, the problem appears similar: only 0.7-2.4\% of the transferred B cells could be detected in the lymph nodes and spleens of the recipients nine days after transfer (164). Study V was performed to investigate whether neonatal ingestion of Ig-containing milk would lead to increased survival of B cell-transplants in B cell-deficient mice.

In brief, neonatal \( \mu^{-/-} \) mice were either transferred to lactating \( \mu^{+/+} \) foster dams, allowing them to suckle IgG-containing milk, or were kept with their \( \mu^{-/-} \) mothers without IgG in the milk. After adoptive transfer of spleen cells as adults, serum-IgG levels and the numbers of Ig-secreting cells, T and B cells were determined. The results showed that the mice, which had suckled IgG-containing milk had significantly higher levels of serum-IgG and splenic B cells, as well as higher number of Ig-secreting cells in spleen and bone marrow. Surprisingly, the numbers of B220\(^+\) cells was low in both
groups of mice. It would have seemed more likely to predict that the difference in B cell-number between the two groups would have been greater, since there was a marked difference in both Ig-secreting cells and serum-IgG levels. Interestingly, the same type of pattern, i.e. very low levels of B cells despite high numbers of Ig-secreting cells and serum-IgG levels, were observed in \( \mu^{-/-} \) subjected to neonatal adoptive transfer of spleen cells (Paper IV). One reason for this could be that the differentiation of the transferred B cells was, somehow, skewed or biased towards plasma cell development. Another possibility would be that the detected Ig-secreting cells actually were already, or were determined to become, plasma cells at the time of transfer.

In summary, this study (paper V) showed that neonatal suckling of IgG-containing milk dramatically affects the outcome of spleen cell transfers to non-irradiated adult B cell-deficient mice. Since it was observed in paper IV that maternal B-lineage cells engraft B cell-deficient offspring, it could be argued that such cells could be responsible for the observed effects in this study (paper V). It seems more likely, however, that the maternal IgG, which could be expected to have a systemic distribution, would act as a more efficient tolerising antigen than the few cells that occasionally engraft the neonate.

**ABSENCE OF MATERNAL MILK-Ig AFFECTS THE SERUM-IgG LEVELS AND THE IMMUNE RESPONSES IN NORMAL MICE**

This study (paper VI) was performed to investigate how the absence of postnatally transmitted antibodies affects the offspring. Normal C57BL/6 pups were either kept with their own mothers, or transferred to lactating B cell-deficient foster mothers directly after birth. The experimental group consequently lacked postnatal transmission of IgG (Ig\(^{-}\) group), while the control group ingested milk that contained IgG (Ig\(^{+}\) group). The experiments were carried out in two environments: a conventional animal house environment with a relatively high pathogen pressure and a SPF environment, which was free from mouse hepatitis virus, minute virus of mice and Sendai viruses. Serum-IgG levels, as well as numbers of splenic B and T cells were determined at different time points after birth.

The results showed that the unimmunised mice that did not receive maternal IgG via the milk had significantly higher serum-IgG levels, but fewer splenic B cells than the control mice at day 35 of age. This was also the case in the cleaner SPF environment, although the differences were less pronounced. Two other reports have previously used similar experimental
models, although they focused on effects at earlier stages of development (165, 166). Delassus et al reported that maternal Ig-transmission does not affect the rate of maturation of the B cell compartment in foetal mice (165). In contrast, Malanchère and colleagues reported that maternal Ig-transmission has a positive effect on the B cell repertoire in seven day-old progeny, although the IgM production is inhibited (166). The latter appears to be in agreement with the results in paper VI, although a difference in B cell-numbers in seven day-old pups was not observed. It is possible that prenatally transmitted antibodies that had not yet been degraded interfered with the results at this early time point.

Hypothetically, the increased levels of serum-IgG in the offspring that had not experienced postnatal maternal Ig-transmission, could be dependent on one, or a combination of the following possible mechanisms. The clearing of antigens or the blocking of antigenic epitopes could be more efficient in mice that received maternal IgG, (since the maternal IgG could be expected to be directed against common environmental antigens), resulting in fewer cells being primed. High levels of maternal IgG in the neonatal circulation may also negatively affect or regulate the development of plasma cells, since the immediate need for antibody-secreting cells could be expected to be lower if maternal Ig is present. It may also be possible that high levels of maternal IgG negatively influence the life span of the plasma cells. Yet another possibility would be that maternal IgG induces a higher rate of IgG-catabolism.

From our perspective, an increased antigen clearing or blocking appeared to be one of the most likely events leading to the lower IgG-levels in the mice that had experienced maternal Ig-transmission. This was supported by the fact that mice that received maternal IgG-anti-TNP, showed a lower response against TNP after immunisation as adults. In addition, it was recently reported that FcγR-deficient mice show a suppression of the immune response after immunisation with IgG-antigen-complex, indicating that the suppression can be mediated via FcγR-independent mechanisms, such as, blocking of antigenic determinants (23). It cannot, however, be excluded that FcγR-mediated, or Fc-dependent but FcγR-independent mechanisms contribute to the observed effects in our experimental model with unimmunised mice.

Since the transmission of maternal antigen-specific antibodies in the “artificial” immunisation experiment gave rise to a lower immune response, it was of interest to investigate how mice that did not receive any maternal antibodies at all, responded to an immunisation later in life. Interestingly, the mice that lacked maternal Ig-transmission gave a much higher response to adult immunisation with sheep red blood cells, than the
mice that had received maternal IgG. The neonatal transmission of maternal antibodies, even non-specific ones, appeared to give effects on the specific immune response in the adult offspring. This indicated that maternal antibodies, in addition to the antigen clearing, could have an immunoregulatory impact on the immune response of the adult offspring.
CONCLUDING REMARKS

• *In vivo* treatment of pregnant mice with either IFNγ or 5-azacytidine cannot induce placental MHC class II mRNA expression and neither of the treatments affect foetal survival. It is, however, possible to obtain MHC class II molecules at the surface of cells of trophoblast origin *in vitro* as a result of transfections with MHC class II a and b genes.

• Neither foetal, nor maternal production of the Th2-cytokines IL-4 and IL-10 appear to be crucial for the completion of allogeneic pregnancy in mice.

• Maternal B-lineage cells - most probably Ig-secreting cells - occasionally infiltrate B-cell-deficient offspring and stay functional for long periods of time. Although the mechanism for the transmission of maternal cells is unknown, I think that the finding could be of importance in many fields of immunology and developmental biology.

• Neonatal ingestion of Ig-containing milk increases the survival of B-lineage cells after adoptive transfer of spleen cells to non-irradiated adult B cell-deficient mice. This indicates that “cross-fostering” could be used to facilitate long-term reconstitution of Ig-secreting cell numbers in B cell-deficient mice.

• Absence of maternal IgG-transmission resulted in increased serum-IgG levels and a smaller proportion of B cells in normal unimmunised mice. The experiments on antigen-specific IgG-transmission suggested that lack of antigen clearing/blocking could be one reason for the elevated serum-IgG levels. It cannot be excluded, however, that the maternal IgG exerts specific regulatory effects. These could be Fc-dependent and/or FcγR-mediated, since even neonatal transmission of non-specific maternal IgG lowered the immune response after immunisation as adults.
förutsättningarna för att en individ av en art ska överleva varierar och är beroende av ext Levinadssätt, klimat och antalet rovdjur. En förutsättning som dock är gemensam för de flesta arter, beroende av var och hur de lever, är att de måste ha ett effektivt skydd mot infektioner och parasiter. För att uppnå detta har både ryggradslösa djur och ryggradsdjur utrustats med ett immunsystem. Ryggradsdjurens (och då främst däggdjurens) immunsystem är avsevärt mer komplicerat uppbyggda och består av ett intrikat nätverk av olika typer av vita blodkroppar. Dessa samarbetar och signalerar till varandra för att kunna avlägsna främmande partiklar, virusinfekterade celler, tumörceller mm.


I de delarbeten som ingår i min avhandling berörs olika aspekter av immunologiska interaktioner mellan modern och hennes avkomma i möss. I den första delen av avhandlingen (studie I, II och III) har jag undersökt vissa mekanismer som anses vara viktiga för att fostret inte ska stötas bort av mammans immunsystem. Det är känt sedan tidigare att s k transplantationsmolekyler, som spelar en avgörande roll för huruvida ett
transplanterat organ ska stötas bort eller inte, har en ytterst begränsad förekomst i moderkakan. Just den typ av transplantationsmolekyl som jag har studerat (studie I och II) finns normalt inte i moderkakan. För att bringa klarhet i huruvida absaknad av den molekyl är avgörande för fostrets överlevnad, gjordes försök att stimulera fram dessa molekyler i moderkakan. Behandlingen med substanser, som i andra vävnader effektivt stimulerar förekomst av transplantationsmolekyler, har inte samma verkan i moderkakan. Detta tyder på att speciella regleringsmekanismer föreligger.

Fördelningen och balansen mellan vissa typer av lösliga signalämnen i moderkakan, s k cytokiner, anses också vara av stor vikt för fostrets överlevnad. I studie III studeras möss, som via genmanipulation saknar två av de mest omtalade cytokinerna (IL-4 och IL-10). Studien visar att ingen av de nämnda cytokinerna är absolut nödvändig för fostrens överlevnad, eftersom levande ungar föds efter till synes normala graviditeter.

I den senare delen av avhandlingen (studie IV, V och VI) studeras överföring av antikroppar från mamman till avkomman och olika effekter av denna överföring på avkommans immunsystem. I studie IV har immundefekta möss som inte kan producera antikroppar studerats (de saknar s k B-celler). Transgena grön-fluorescerande honor användes också som mammor i försöken. Dessa möss har via genmanipulation fått ett protein från en manet som gör att alla celler i musen lyser i grönt (!) om man placerar musen i UV-ljus. Om de immundefekta ungarna hade diat en "grön" mushona, kunde man hitta funktionella antikroppsproducerande celler som härrör från mamman (cellerna var gröna) i ungarna. Detta ger nya intressanta perspektiv på överföring av passiv immunitet från modern till avkomman.

Man vet att B-cellstransplantat har dålig överlevnad i möss som saknar B-celler. Studie V visar att om dessa möss diar en normal hona när de är unga, accepterar de lättare de transplanterade B-cellerna när de är vuxna. Detta skulle kunna underlätta studier av vad som händer med transplanterade B-celler.

Resultaten från studie VI visar att normala musungar som inte fått antikroppar via modermjölk, reagerar med ett mycket kraftigare immunsvar mot ett injicerat ämne. Detta tillsammans med att de här ungarna har en något annorlunda fördelning av vita blodkroppar, tyder på att mammans antikroppar inte bara skyddar ungen genom att avlägsna främmande ämnena, utan också har en immunreglerande funktion.
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