HUMANIZED MICE AS A MODEL TO STUDY HUMAN IMMUNITY

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Stockholm 2012
“Success is not final, failure is not fatal: it is the courage to continue that counts”

Sir Winston Leonard Spencer-Churchill
ABSTRACT

Mice with human immune systems have evolved over the last three decades. Over the years, these humanized mouse models have provided us with valuable information on human immunity. Recent developments in recipient mouse strains and engraftment protocols have resulted in models with high level of de novo formation of human immune cells. Here we describe the development and improvements of humanized BALB/c/Rag2−/−/IL2Rγc−/− and NOD/SCID/IL2Rγc−/− mice and employ such mice or studies on various aspects of human immunity.

We show that engraftment with human cells depends on the recipient strain and conditioning regiment. While we confirm that T cells that developed in the mouse are functional and respond to allogeneic cells and mitogens in vitro, no rejection was observed in vivo after transplantation of human β-islets under the kidney capsule of humanized mice (Paper I).

In a second study, we showed that human CD56dim NK cells in humanized mice and in recipients of a bone marrow transplant are subject to further differentiation. We observed that CD57 and killer cell immunoglobulin-like receptors (KIRs) are acquired during differentiation of CD56dim cells. (Paper II)

Infection studies in humanized mice have thus far been almost exclusively limited to infections with pathogens specifically targeting human immune cells. We explored the use of humanized mice for mycobacterial infections, which are not restricted to infection of human cells. We found that humanized mice contained higher bacterial titers in comparison to controls. While this finding could be attributed to dysfunctional T cell responses and impaired anti-mycobacterial responses by human macrophages, we found that humanized mice infected with Mycobacterium bovis BCG or Mycobacterium tuberculosis developed organized granulomas similar to those found in humans. Furthermore, we demonstrated that human CD4+ cells impair mycobacterial control but are essential for the development or maintenance of granulomas (Paper III).

Finally, we used humanized mice to shed light on Epstein Barr Virus induced latency. Infections with this human specific virus resulted in a CD4/CD8 T cell ratio skewed towards CD8 and the differentiation of T cells from naïve to effector memory cells. A variable number of infected mice showed tumors and B-cell proliferation in vivo. In vivo depletion of CD8+ cells increased the frequency of tumors and ex vivo proliferation of transformed B cells. Surprisingly, ex vivo proliferation of B cells from CD8+ cell depleted or non-depleted mice was inhibited in presence of cyclosporine A, suggesting that CD4+ T cells exerted a supporting effect on cells displaying a latency type which otherwise would not proliferate in vitro. This finding was confirmed by analysis of viral promoters in CD4+, CD8+ and non-depleted infected animals (Paper IV).

In conclusion, our studies show that current humanized mouse models can be used to improve our knowledge of various aspects of human immunity, such as alloreactivity, the ontogeny of hematopoietic cells, the immunobiology of human-lymphoid specific as well as non-species specific microorganisms and the regulation of granuloma formation in infectious and non-infectious diseases.
LIST OF PUBLICATIONS

I. Alloreactivity but failure to reject human islet transplants by humanized BALB/c/Rag2-/-γc-/- mice
   Stella Jacobsson, Frank Heuts, Julius Juaréz, Monica Hultcrantz, Mattias Svensson, Martin Rottenberg and Malin Flodström-Tullberg

II. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56<sup>dim</sup> NK-cell differentiation uncoupled from NK-cell education

III. CD4<sup>+</sup> cell-dependent granuloma formation in Mycobacterium bovis BCG-infected humanized mice
    Frank Heuts, Dolores Gavier-Widén, Berit Carow, Julius Juarez, Hans Wigzell and Martin E Rottenberg
    Manuscript

IV. T-cell dependent regulation of EBV latency in humanized mice
    Frank Heuts, Martin Rottenberg, Daniel Salamon, Abu Rasul, Monika Adori, Eva Klein and Noemi Nagy
    Manuscript
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<tr>
<td>AGM-region</td>
<td>Aorta-Gonad-Mesonephros region</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>BCR</td>
<td>B-Cell Receptor</td>
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<tr>
<td>BLT mice</td>
<td>Bone marrow-Liver-Thymus mice</td>
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<tr>
<td>BRG</td>
<td>BALB/c/Rag2&lt;sup&gt;−/−&lt;/sup&gt;/IL2Rγ&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>CB</td>
<td>Cord Blood</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
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<td>CMP</td>
<td>Common Myeloid Progenitor</td>
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<tr>
<td>CMV</td>
<td>Cytomegalo Virus</td>
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<tr>
<td>CB</td>
<td>Cord Blood</td>
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<td>CMV</td>
<td>Cytomegalo Virus</td>
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<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
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<tr>
<td>EBNA</td>
<td>EBV Nuclear Antigen</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>GC</td>
<td>Germinal Center</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>HIS mice</td>
<td>Human Immune System mice</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
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<td>HSCT</td>
<td>Hematopoietic Stem Cell Transplant</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL-</td>
<td>Interleukin-</td>
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<tr>
<td>IM</td>
<td>Infectious Mononucleosis</td>
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<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<tr>
<td>KIR</td>
<td>Killer cell Immunoglobulin like Receptor</td>
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<tr>
<td>LCL</td>
<td>Lymphoblastoid Cell Line</td>
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<td>Abbreviation</td>
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<tr>
<td>LMP</td>
<td>Latent Membrane Protein</td>
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<td>LPD</td>
<td>Lymphoproliferative disease</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>M. bovis</td>
<td>Mycobacterium bovis</td>
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<tr>
<td>M. tuberculosis</td>
<td>Mycobacterium tuberculosis</td>
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<tr>
<td>MDR</td>
<td>Multi-Drug Resistant</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>NK cell</td>
<td>Natural Killer cell</td>
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<td>NOD</td>
<td>Non-Obese Diabetic</td>
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<td>NSG</td>
<td>NOD/SCID/IL2Rγ−/−</td>
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<tr>
<td>PBL</td>
<td>Peripheral Blood mononuclear Leukocytes</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptors</td>
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<tr>
<td>PTLD</td>
<td>Post-Transplant Lymphoproliferative Disease</td>
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<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
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<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
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<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>T1D</td>
<td>Type 1 Diabetes</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
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<tr>
<td>XDR</td>
<td>Extreme-Drug Resistant</td>
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1 INTRODUCTION

Laboratory mice have been of instrumental importance in our current understanding of immunology. Their small size, rapid reproduction rate, genetic similarity to humans and possibilities to modify genes and proteins make for a very good model to expand our knowledge. While their importance is obvious, mice are not humans and therefore limitations in the translatability of data found in mice do occur. For these reasons other experimental models, notably primate models, are in use. These models are more laborious, expensive, more variable and limited when it comes to modifications. Hence, a small animal model that, at least in part, more closely resembles humans would be welcome.

Mice engrafted with human immune cells have been used to study human immune responses and human immune responses in mice for the last three decades. During this period such models have been improved and they have been used for an increasing array of studies. In this thesis the term “humanized mouse” is used for mice carrying human immune cells.

In the work presented here I describe the generation of such mice and determine their applicability in different studies on human immune cells in vivo.

1.1 IMMUNE CELLS AND THEIR DEVELOPMENT

1.1.1 Early development

Cells from both the innate and adaptive immune system develop from hematopoietic stem cells in a sequence of processes which are tightly regulated by a multitude of soluble molecules, including growth factors, cytokines and chemokines (Figure 1). In mammals this process moves from the yolk sac via the aorta-gonad-mesonephros (AGM)-region to the fetal liver in fetal and neonatal life and is finally established in the bone marrow in adult life (reviewed in 1). In addition to these sites, hematopoietic stem cells have been found in the placenta and umbilical arteries 2-4. During their development from stem cells to specialized immune cells, these cells pass through various progenitor stages during which they gradually loose their capacity to self renew. The CD34 molecule is expressed on the majority of hematopoietic cells that harbour repopulating capacities including CD34⁺CD38⁻ hematopoietic stem cells, and
Figure 1: Overview of human hematopoiesis.
1.1.2 Innate immune cells

Cells of the innate immune system can recognize a large variety of common structural patterns of microorganisms via germline encoded pattern recognition receptors (PRRs). Granulocytes and monocytes/macrophages are able to phagocytose and kill microbes in their phagosomes and lysosomes using toxic compounds and enzymes.

Dendritic cells form the link between the innate and adaptive immune system. After activation at a site of infection they migrate to draining lymph nodes where they present antigen to T and B cells, thereby initiating the adaptive immune response.

1.1.2.1 NK cells

Natural killer cells (NK cells) are cytotoxic lymphocytes, which are able to distinguish between healthy and unhealthy cells in the same individual as well as between congenic and allogeneic cells. As such, they have a role in the defence against tumors and infection. They develop from the common lymphoid progenitor (CLP) in the bone marrow under influence of IL-2 and/or IL-15. In peripheral tissues they can be divided into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells, based on their expression of the adhesion molecule CD56. The former of these two populations is considered to be less mature and they are further characterized by absence of the Fc receptor CD16 and low or no presence of inhibitory killer cell immunoglobulin like receptors (KIRs). CD56\textsuperscript{dim} cells on the other hand do express CD16 as well as KIRs.

Unlike other immune cells, elimination of target cells by NK cells is based on the lack of inhibiting signals delivered by receptors recognizing self molecules. This mechanism was postulated in 1981 as the missing self hypothesis and proven several years later. NK cell activation depends on the interaction of a variety of activating and inhibitory receptors on the surface of the NK cell with their respective ligands on a potential target cells. The net signal of these interactions is decisive on whether the NK cell will kill the target cell or not. Inhibitory receptors, including most KIRs, recognize MHC class I molecules, which are expressed by most healthy cells in the body. Activating receptors on the other hand recognize ligands that are upregulated in cells that have undergone stress, have been infected by a microbe or have undergone malignant transformation. Although ligands for activating receptors can be expressed by healthy cells as well, the input of the inhibitory receptors will prevent the
NK cell from becoming activated and killing the target cell. In ailing cells the expression of MHC class I molecules is often down regulated or absent, decreasing the chances of recognition and elimination by CD8\(^+\) cytotoxic T cells, but tilting NK cells towards an activated state\(^{21,22}\). Activated NK cells will release pro-inflammatory cytokines like interferon-\(\gamma\) (IFN-\(\gamma\)), Tumor Necrosis Factor-\(\alpha\) (TNF-\(\alpha\)) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and kill target cells through release of vesicles containing perforins, and granzymes\(^7\).

Surprisingly, mice lacking MHC class I molecules have NK cells that are not autoreactive. The reason for this lies in the finding that NK cells need to be licensed or educated (hereafter called NK cell education) before they have the ability to kill target cells\(^{23}\). This education process relies on the acquisition of at least one KIR recognizing a self-MHC-I molecule\(^{23,24}\). If this does not occur NK cells become inert. This indicates that NK cells are subject to change in functionality and phenotype during their lifespan. Findings presented in this thesis and by others further illustrate the differentiation of CD56\(^{dim}\) cells.

1.1.3 Cells of the adaptive immune system

Together with B- cells, T-cells form the adaptive immune system. B cells develop from the common lymphoid progenitor in the bone marrow and become antibody-producing cells in the periphery\(^7\).

After the initial development in the bone marrow, T cell precursors migrate to the thymus where they will develop into functional mature T cells. In the initial stages of T cell maturation progenitors acquire a random T cell receptor through rearrangement of gene segments. This process involves a number of molecules including the RAG-1 and -2 proteins as well as Prkdc (a DNA-dependent serine threonine protein kinase). Lack of any of these proteins thus results in failure to produce mature T and B cells, since the receptor rearrangement is mediated by the same proteins in B cells as well\(^7\).

As receptor rearrangement is random, T cells expressing receptors are selected depending on their ability to recognize antigen-MHC complexes on thymic epithelium cells and bone marrow derived antigen presenting cells (APC). Therefore, developing T cells expressing CD4 and CD8 molecules on their surface will undergo a two step selection process in the thymus\(^{25}\): first, cells expressing receptors capable of
recognizing complexes of host derived antigen in native MHC class I or class II molecules expressed on thymic epithelial cells are selected and loose expression of CD4 or CD8 respectively. Cells unable to recognize such complexes will undergo apoptosis. After positive selection, T cells whose receptors have strong affinity for self-MHC-peptide complexes on bone marrow derived APC (DCs and macrophages) are eliminated by apoptosis as well. Only cells passing this selection process survive and migrate to secondary lymphoid organs as naïve cells and become activated after recognition of cognate peptide presented in MHC molecules of dendritic cells that simultaneously express co-stimulatory molecules like CD80, CD86 and CD40 which are required for activation of the T cell.

1.1.3.1 Types of T cells

Based on the recognition of MHC class II or class I T cells can be divided into CD4+ T helper (T_h) cells and CD8+ cytotoxic T (T_c) cells. The latter will recognize peptides presented by MHC-I molecules on dendritic and target cells. Recognition of peptides derived from viral proteins can result in the lysis of infected cells through release of vesicles containing granzymes, perforins and FAS ligand.

At present, four subsets of CD4+ T cells have been described in vivo based on their cytokine profile and transcription factors. T_h1 cells using the T-bet transcription factor produce cytokines like IFN-γ, and are important in the control of intracellular bacterial and protozoal infections. Th2 cells on the other hand are characterized by the use of the GATA-3 transcription factor and the production of cytokines like IL-4, IL-5 and IL-10, which mediate immune responses towards extracellular pathogens such as worms. A third type of CD4+ T cell are T_h17 cells, which use RORγT and produce IL-17, which causes inflammation and recruitment of leukocytes. Th17 cells are critical for the control of fungal infections. Unlike the aforementioned types of CD4+ T cells, the function of regulatory T cells (T_reg) is to dampen immune responses in order to prevent immune-mediated inflammatory damage. They perform this action via the release of anti-inflammatory cytokines TGF-β and IL-10 and use FoxP3 as characteristic transcription factor.
1.1.3.2 T cells in transplantation immunology

While proper selection in the thymus prevents T cells from attacking self cells, selection does not eliminate cells that are able to recognize MHC-peptide complexes on cells from transplanted organs. The consequence of this is that allogeneic organ transplants are prone to be rejected by the immune system if no preventive measures are taken. T cell mediated rejection can be either acute or chronic. Alloreactive T cells and antibodies mediate acute rejection shortly after transplantation. Recognition of allo-antigens results in killing of target cells by CD4$^+$ and CD8$^+$ T cells, while antibody deposition on cells from the graft results killing via the complement system $^7$.

In chronic rejection, usually observed in MHC-matched transplants, graft destruction is caused by cytokines derived from antigen specific CD4$^+$ T cells after activation by APCs in the graft. These cytokines activate macrophages and thereby induce a delayed type hypersensitivity (DTH) response which leads to damage of vessel walls in the graft and subsequent smooth muscle cell proliferation which in turn leads to occlusion of the vessel and reduced blood flow to the transplant $^7$. Given the importance of T cells in graft rejection, measures to prevent rejection are focused on suppressing T cell function. The introduction of treatment with T cell suppressive drugs like cyclosporine A in the early 1980s has resulted in a marked increase in graft longevity $^7$.

1.2 TUBERCULOSIS

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). It is estimated that one-third of the world’s population is infected with the bacteria $^{26}$. However, in the vast majority of infected individuals the bacteria are kept in check for life by the host’s immune system and no active disease develops. These individuals are healthy but remain chronically infected. In 5 to 10% of newly or chronically infected individuals disease does develop which results in an annual death toll of approximately 2 million $^{27,28}$. Over the last decades the incidence of active TB has increased and this has only recently come to a stand still $^{26}$. The steep increase has been attributed to co-infections with HIV, which greatly enhances the risk to develop active disease due to impaired immune responses $^{28}$, and the appearance of multi-drug resistant (MDR) and extreme-drug resistant (XDR) strains of *M. tuberculosis* $^{29}$. In addition, the available vaccine, *Mycobacterium bovis*-bacillus Calmette-Guérin (BCG) has been in use for
over 90 years in humans, does not show reliable protection against pulmonary TB \(^{30,31}\), which is the most prevalent form of the disease in adults.

1.2.1 Mycobacterial infection and immune responses

*M. tuberculosis* infects pulmonary cells after inhalation of aerosol droplets carrying the bacterium \(^7\). In the lung bacteria are ingested by alveolar macrophages, which are activated through recognition of bacterial cell wall components by pattern recognition receptors (PRRs) like Toll Like Receptors (TLRs) and NOD receptors \(^{32-35}\). Activated macrophages release pro-inflammatory cytokines and chemokines, which recruit and activate neutrophils, macrophages and NK cells to the site of infection \(^{36,37}\). Dendritic cells activated after taking up mycobacteria will migrate to the draining lymph node where they can in turn activate antigen-specific CD4\(^+\) and CD8\(^+\) T cells which will be exported to the periphery and recruited to the site of infection. These T cells can activate microbicidal mechanisms of macrophages through the release of IFN-\(\gamma\) or by killing the infected cells \(^{38-40}\).

1.2.2 Granuloma

Despite the potent specific immune responses it generates, *M. tuberculosis* has developed mechanisms to dampen the immune responses which prevent the immune system from eradicating the infection \(^{41}\). Phago-lysosome fusion and the acidification of phagosomes are blocked by *M. tuberculosis* and the infection also inhibits IFN-\(\gamma\) mediated activation of macrophages \(^{42}\). As a consequence of these actions the bacteria can survive in macrophages, which leads to chronic antigen stimulation. This leads to recruitment of more inflammatory cells, notably macrophages and antigen-specific T cells, which leads to the formation of granulomas \(^{43,44}\). The granuloma form a microenvironment shielded from surrounding tissue in which there is a balance between mycobacterial growth and killing as infected macrophages are in close proximity of surrounding T cells. In humans, granulomas are composed of a core of macrophages, some of which differentiate into multi-nucleated giant cells whereas others turn into epithelioid cells. Surrounding this macrophage core is a cuff of antigen specific T cells, the majority of which is CD4\(^+\). A fibrotic cuff surrounding the lesion and caseous necrosis of macrophages forming a caseous center characterizes well-developed granulomas \(^{45}\).
The role of the granuloma in host-pathogen interaction is subject to debate. Most data suggests that mycobacteria are contained in the granuloma. In support of this idea, TB is more prone to be reactivated in latently infected individuals who are immunosuppressed like for instance HIV infected individuals. In these individuals the granuloma structure is lost. Furthermore, rheumatoid arthritis patients that are treated with antibodies against TNF-α, a cytokine essential for proper granuloma formation, have also been found to reactivate TB. Recent findings in a zebrafish model of granuloma formation suggests however that the granuloma is a ‘safe haven’ where mycobacteria can replicate relatively unharmed after which they can move out of the granuloma in an infected macrophage and form a new granuloma in a distal site. Therefore, models that would allow for increased understanding of granuloma development and maintenance are welcome to shed more light on the immunological function of this structure.

1.2.3 Current mouse model for Tuberculosis

As mycobacteria are not confined to infecting one species, much of the information found in the previous paragraphs is based on studies in animals. It is therefore not surprising that most of the currently available information is based on studies in mice. Indeed, there is a resemblance in many key features of the human and mouse protective immune responses to TB including the role of CD4+ T cells, IFN-γ and TNF-α. Upon closer examination differences become evident though. Mouse granulomas do not show the caseous necrosis that is characteristic for human granulomas. Also, granulomas are less sharply defined and the relative location of T cells and macrophages is different. Giant cells, derived from fusion of epitheloid macrophages and the fibroblast-derived collagen capsule are absent in the mouse. Another important point is that while most infected humans remain healthy for years, all infected mice will show development of active disease.

In addition to the mycobacteria specific immune responses, there is also a growing need to understand the role of HIV co-infections in the reactivation of TB. Unlike mycobacteria, HIV only can infect human cells, impeding the use of mice as an experimental model. Therefore, new models overcoming the limitations listed above are needed.
1.3 EPSTEIN-BARR VIRUS

Epstein-Barr Virus (EBV), a human specific virus that belongs to the herpes viruses, was first found in a Burkitt’s lymphoma cell line \(^{52,53}\). Shortly after its discovery it was shown that EBV was capable of transforming B cells \textit{in vitro} \(^{54,55}\). Apart from Burkitt’s lymphoma EBV has also been associated to Hodgkin lymphoma, post-transplant lymphoproliferative disorders (PTLD), NK and T cell lymphomas and nasopharyngeal carcinoma \(^{56}\). Despite these associations, EBV is not the sole causative agent of these tumors, as EBV negative forms of all these tumors have been described. More importantly, EBV is carried by 95% of the world’s population and is harmless in most individuals \(^{57}\). Generally, primary infection occurs in early childhood and is asymptomatic. Individuals that contract the virus in early adulthood or later can develop a self-limiting disease known as infectious mononucleosis (IM) or kissing disease due to its oral transmission. This disease is characterized by massive expansion of NK cells and CD8\(^+\) T cells and consequently lymphadenopathy, hepatitis and splenomegaly \(^{58-60}\).

1.3.1 EBV latency

As mentioned above, EBV is harmless in the vast majority of individuals carrying the virus, as it is present in a latent form in B cells which are kept under control by the immune system. In latently infected cells the viral genome is contained in a circular episome \(^{61}\). Based on the expression of 9 latent genes, EBV Nuclear Antigens (EBNA) 1-6 (also known as 1, 2, 3A, 3B, LP and 3C) and Late Membrane Proteins (LMP) 1, 2A and 2B, several types of latency have been classified. Two untranslated RNAs, the Epstein-Barr encoded RNAs (EBERs), are expressed in all latency types. \textit{In vitro} infection of B cells gives rise to highly proliferative lymphoblastoid cell lines (LCLs), which are immortalized B cells expressing a type III latency in which all 9 latent proteins are expressed. Based on PCR data, a model for the establishment of EBV latency \textit{in vivo} has been proposed \(^{62}\). In this model, naïve B cells are transformed by EBV and turn into type III latency expressing cells. Specific cytotoxic CD8\(^+\) and CD4\(^+\) T cells eliminate most of these rapidly proliferating and highly immunogenic cells, but some escape to germinal centers (GCs) in lymph nodes. During this process the expression of latent proteins is limited to EBNA1 and the LMPs...
which is the signature of latency II. LMP1 mimics a constitutively active CD40 and mediates passage through the GC without T cell help. LMP2A mimics B cell receptor (BCR) signalling and allows for survival of infected B cells in the GC without recognition of cognate antigen. In addition, LMP1 down regulates Bcl-6 and in doing so allows entry of infected B cells into the memory B cell pool. Here only EBNA1 (latency I) or no latent proteins are expressed (latency 0) and the infected cells become invisible to the immune system. EBV occasionally enters the lytic cycle, which results in the production of new virions.

Cells expressing other than type III latency do not spontaneously proliferate in vitro. These latency types are associated to different malignancies like Burkitt’s and Hodgkin’s lymphoma though. In vitro studies on Burkitt’s and Hodgkin’s lymphoma derived cell lines revealed that IL-4, -10, -13 and -21 could induce a shift from latency I to latency II, whereas IL-21 and CD40L promoted the transition from latency III to II. Since EBV only infects human cells studies on in vivo models are very limited, but the use of humanized mice, which is discussed below, has allowed for new insights in EBV immunobiology.

1.4 EVOLUTION OF HUMANIZED MICE

1.4.1 Development of recipient mouse strains

As immunocompetent mice will reject xenogeneic transplanted cells, studies on human immunity in mice have heavily relied on the development of immunocompromised mice. The first step towards generating mice with human immune responses was made with the discovery that human immune cells were able to engraft mice carrying a spontaneous mutation in Prkdcscid in the early 1980s. In these SCID mice B and T cell development was severely impaired which prevented them from rejecting transplanted human cells. While this was a major step in the development of humanized mice, there were many limitations. The engraftment level of human cells was low and de novo formation of human immune cells was absent. As Prkdc is involved in DNA repair the mice were also extremely radiosensitive. Furthermore, over time some mouse B and T cell populations would appear in these mice, a phenomenon known as leakiness. These problems could be circumvented in mice carrying targeted deletions for RAG-1 or RAG-2, which, as is the case for Prkdc, are involved in formation of antigen receptors on B and T cells.
However, a problem that could not be resolved by mutating the RAG genes was the high NK cell activity in these mice, which impaired the grafting of human cells\textsuperscript{75,76}. It was found that some strains generated by crossing of CB17/SCID mice with various other mouse strains were better recipients of human hematopoietic cells than others\textsuperscript{77}. This difference was attributed to a variation in NK-cell activity levels between them. NOD mice carrying the Prkdc\textsuperscript{−/−} mutation (NOD/SCID mice) had particularly low NK cell activity and proved to be better recipients for human hematopoietic cells than other strains. In addition, the phagocytes system of NOD/SCID mice also had defects, and this phenotype aided in the tolerance of mice to human grafts\textsuperscript{78-80}. Although NOD/SCID mice were better in supporting human grafts than SCID mice, they were not completely devoid of NK cell activity and on top of that their life span was short as they were prone to develop thymic lymphomas\textsuperscript{77}. Despite a variety of additional mutations on this strain there were no big improvements on the front of human cell or tissue engraftment until the generation of NOD/SCID mice in which the IL-2 receptor gamma-chain was rendered non-functional through targeted mutation\textsuperscript{81-84}. The IL2R\textgammachain is the common signalling subunit in the receptors for IL-2, IL-4, IL-7, IL9- IL-15 and IL-21 and deficiency of this molecule results in defective lymphoid development\textsuperscript{85}. However, signalling via this receptor is critical for NK cell development\textsuperscript{86}. Generation of either NOD/SCID or BALB/c/RAG-2\textsuperscript{−/−} mice lacking the IL2R\gamma resulted in strains completely deficient in mouse B, T and NK cells.

At present, NOD/SCID/IL2R\gamma\textsuperscript{−/−} (NSG) or BALB/c/RAG-2\textsuperscript{−/−}/IL2R\gamma\textsuperscript{−/−} (BRG) mice are the standard recipients for generation of humanized mice. NSG mice have shown stronger engraftment\textsuperscript{87}. Although one difference with BRG mice is the impaired macrophage function in NSG mice, it has recently been shown that the homology to the human signal-regulatory protein alpha (SIRP-\alpha) in these mice is responsible for the difference in engraftment potential between them\textsuperscript{88-90}. SIRP-\alpha on phagocytic cells interacts with CD47 on hematopoietic cells and proper interaction results in decreased phagocytosis of target cells\textsuperscript{91}. SIRP-\alpha of NSG mice is more homologous to its human counterpart in comparison to the same protein in BRG mice\textsuperscript{90}, as a result of which less human hematopoietic cells are phagocytized and more cells can engraft the mouse.
1.5 CURRENT HUMANIZED MOUSE MODELS

1.5.1 Hu-PBL-SCID

Studies in CB17/SCID mice showed that an easy and relatively fast way to study human immunology in mice was to inject peripheral blood mononuclear leukocytes (PBL) into these mice. These cells will repopulate the mice and allow for studies on human tropic diseases such as HIV, Dengue virus and EBV \textsuperscript{92-95}. Although recall responses have been observed in these mice, primary responses are hardly, if at all present \textsuperscript{96,97}. Furthermore, in this model T cells are educated on the thymus of the human donor and are therefore HLA restricted. A direct consequence of this HLA specificity is that human T cells can cause a xeno-GVHD reaction \textsuperscript{98,99}, as a result of which long term studies of the human immune system in this model are limited.

The model has however been shown very useful for studies on acute graft rejection \textsuperscript{100}. Over time and using various approaches, human skin allografts and human islet allografts have been studied successfully in Hu-PBL mice. The elimination of murine NK cells in BRG and NSG mice has allowed for stable systems to study the role of human T cells after co-transplantation of human PBMCs and allogeneic tissues like skin or β-islets \textsuperscript{101-104}.

1.5.2 HIS mice

More sophisticated models rely on the transplantation of human CD34\textsuperscript{+} hematopoietic stem cells. Such cells can be derived from cord blood, fetal liver or peripheral blood after GM-CSF induced mobilization from the bone marrow. Although cells derived from fetal liver are more immature and more abundant, allowing for generation of more mice from the same donor, ethical constraints limit their availability. Cord blood is more easily available and transplantation of CD34\textsuperscript{+} cells derived from this source still results in strongly engrafted mice. Mobilized CD34\textsuperscript{+} cells from adult individuals are less potent than the other options \textsuperscript{105}.

Sub-lethally irradiated BRG or NSG mice transplanted with human CD34\textsuperscript{+} cells show multilineage reconstitution consisting of B and T cells, NK cells, myeloid and plasmacytoid dendritic cells and macrophages \textsuperscript{84,106}. Engrafted mice, designated Humane Immune System (HIS)-mice, furthermore show formation of primary and
secondary lymphoid organs. The majority of the human cells are B and T lymphocytes. Development of the latter cell type depends on the age at which the mice are transplanted. If CD34+ cells are delivered intravenously to adult mice only few T cell develop whereas intrahepatic transplantation into new-born mice results in higher levels of T cell reconstitution. The fact that T cells develop at all in the mouse thymus can be called surprising given the positive and negative selection based on interaction between the T cell receptor and MHC on thymic epithelial and bone marrow derived APC cells respectively. An explanation for T cell development without generation of xenoreactivity can be found in studies describing that non-epithelial cells or hematopoietic cells can also mediate the positive selection. The work discussed later in this thesis was performed on this model.

![Figure 2: Schematic overview of the generation of HIS mice](image)

1.5.3 BLT mice

In order to circumvent the issue of T cell education in the mouse thymus, McCune et al. transplanted a combination of human fetal liver, thymus and lymph node under the kidney capsule of CB17/SCID mice, which gives rise to a human organoid that provides a microenvironment capable of supporting human T cell education. Although human T cells are abundant in this model, they are mainly confined to the organoid and thus reconstitution of other organs is poor.
As it was shown that transplantation of human CD34<sup>+</sup> cells in adult NOD/SCID mice resulted mainly in systemic B cell development<sup>76,95</sup>, models in which mice first received a transplant of human fetal liver and thymus and were subsequently injected with human CD34<sup>+</sup> cells derived from the same fetal liver proved to be very promising. In such mice, designated Bone marrow-Liver-Thymus (BLT-) mice, a functional human immune system with both systemic human T and B cell repopulation developed<sup>113,114</sup>. Since T cells develop in a syngeneic thymus-like structure, the T cell selection is proper in this model. However, as this model requires human fetal material, ethical constraints can form a major hurdle in maintaining this model.

1.5.4 Improving reconstitution of existing models

Although the models described above allow for reconstitution with human immune cells, a lot of effort has been put into further improving these models in order to make them more representative of the human immune system in terms of ratios of different immune cells and their function.

Several strategies aimed at improving the initial engraftment of CD34<sup>+</sup> cells. One approach has been the manipulation of CD34<sup>+</sup> cells <em>in vitro</em> prior to transplantation through expansion in presence of different growth factors, cytokines and/or lentiviral transduction<sup>115-118</sup>. Additionally, the effect of different routes of administration of HSCs has been investigated<sup>81,83,119,120</sup>. On the other hand, alternative ways for conditioning of the recipient mice have been proposed<sup>121</sup>. Although promising results have been obtained, transplantation of non-manipulated cells in mice conditioned by sublethal irradiation is still the standard.

Various approaches have led to improvements in the repopulation of different types of human immune cells. Development and frequency of human T cells improved after transplanted mice were treated with human IL-7 and SCF or after <em>in vivo</em> lentiviral vector delivery of human IL-7<sup>122-124</sup>. In other studies the genetic manipulation of the recipient mice by knocking in human cytokine gene has been explored. NSG mice expressing human GM-CSF, SCF and IL-3 showed markedly elevated levels of functional FoxP3<sup>+</sup> T<sub>regs</sub><sup>125</sup>, whereas BRG mice expressing human IL-3 and GM-CSF, M-CSF or thrombopoietin showed improved reconstitution of alveolar macrophages, macrophages or overall engraftment respectively<sup>126-128</sup>. Improved myeloid differentiation was also observed in mice with membrane bound SCF<sup>129</sup>. Transiently
elevated levels of NK cells, DCs, macrophages and red blood cells (RBCs) were observed after hydrodynamic delivery of plasmids expressing selected cytokines\textsuperscript{130}. Although one report suggested otherwise\textsuperscript{83}, mature RBCs are generally not observed in humanized mice. It has been suggested that RBCs are phagocytosed by mouse macrophages as mice depleted of macrophages displayed both human and mouse RBCs in their blood\textsuperscript{131}.

1.5.5 NK cells in humanized mice

Several groups used HIS mice to study NK cells. All subsets of NK cells occur at low frequency in the thymus, liver and spleen but hardly in the bone marrow of humanized mice\textsuperscript{132}. Similar to NK cells in human cord blood, NK cells in humanized mice require preactivation by IL-15 in order function similarly to human adult peripheral blood human NK cells\textsuperscript{133}. The low frequency of human NK cells was explained by the finding that murine IL-15, which is secreted by both hematopoietic and non hematopoietic cells, does not cross react with the human IL-15 receptor. In humanized BRG mice, administration of a complex of human recombinant IL-15 resulted in increased numbers and frequencies of NK cells\textsuperscript{132,134}. Notably, as a result of this treatment the less mature CD56\textsuperscript{bright}-CD16 negative NK population is converted to CD56\textsuperscript{dim}-CD16 positive cells, indicating that the treatment results in maturation of NK cells\textsuperscript{132}. In line with this, transient \textit{in vivo} expression of IL-15 in NSG mice resulted in an increased frequency of NK cells\textsuperscript{130}. Whether this treatment also induced a switch to CD16 positive cells was not investigated. In terms of functionality, humanized mouse derived NK cells responded to IL-15 with production of IFN-\(\gamma\) and granzyme B. Furthermore, in co-cultures with the NK cell sensitive K562 tumor cell line they responded with IFN-\(\gamma\) production, degranulation and cytotoxicity. This tumor killing function was also observed \textit{in vivo} and dependent on NK cells as depletion of CD56 positive cells inhibited this effect\textsuperscript{135}. In conclusion, based on data presented thus far HIS mice seem to provide a solid model to study human NK cells \textit{in vivo}. 
1.5.6 Humanized mice for studies of human infection

Humanized mice provide a good platform for in vivo studies of pathogens with a tropism restricted to human immune cells. As the discovery of HIV and the development of the first humanized mouse models both occurred in the early 1980s, many studies on the immunobiology of this virus have been performed using these models. Even though human cell engraftment was limited in the initial humanized mouse models, mice reproduced the CD4⁺ T cell depletion observed in humans. These models proved useful to study the efficacy of anti-retroviral drugs 111,136-138.

Over the last decade the HIS and BLT models in which functional T cells formed de novo, have allowed for more detailed and extensive studies on HIV immunobiology. In both models HIV infection could occur via the natural mucosal routes of entry 139,140. Cellular and humoral immune responses have been described in response to infection and the depletion of subsets of CD4⁺ T cells has been also found 141,142. Furthermore, humanized mice have been used successfully in various studies addressing new therapies and vaccination strategies 143-147. Taken together, in the recently developed humanized mouse models HIV infection behaves similar to infection in humans in many regards, making it a solid platform for studies on infection with this pathogen.

Infections with Dengue virus (DENV) have also been performed in humanized mice. Infection with DENV resulted in clinical signs resembling those in humans 94,148,149. Using humanized NSG mice transgenic for HLA-A2 (NSG-A2), Jaiswal et. al. found antigen specific T cells 150,151. As with HIV, antigen-specific antibodies were found in response to the infection 152. In general, antigen-specific antibody responses, if present at all are, very low and generally of the IgM isotype 153. The recently developed HLA-class II NOD/RAG-1⁻/⁻/IL2Rγ⁻/⁻ mice showed improved antibody titers as well as class switching in response to vaccination with tetanus toxoid, which illustrates the importance of help from properly educated CD4⁺ T cells for antibody production in humanized mice 154.

As indicated previously, humanized mice have permitted in vivo studies of EBV infections. Transfer of EBV positive PBMCs into SCID mice resulted in lymphoproliferative disease (LPD) 92, indicating that in these models the infection that is contained in healthy individuals cannot be controlled. Since levels of human T cells in these models were low, these experiments illustrated the importance of functional T cell responses in order to keep EBV transformed B cells in check. As was the case for
HIV, the more recently developed humanized mouse models allowed for better studies of EBV immunobiology. Although LPD can still develop in these models, infection leads to a marked expansion of CD$^8^+$ T cells, which respond by producing IFN-$\gamma$ after restimulation with autologous LCLs. The development of LDP has been associated to dose of the viral inoculum; a low dose of virus ($10^5$ Raji Infectious Units (RIU)) was controlled by the immune system whereas a 10 fold higher dose resulted in EBV-driven tumor formation. As expected, it was demonstrated that T cells control the infection. Depletion of CD3$^+$, CD4$^+$ and/or CD8$^+$ T cells resulted in diminished control and frequent tumor formation. Although specific T cell responses were detected, these were mainly directed towards subdominant EBV epitopes. In order to find immunodominant T cell epitopes Strowig et. al. used NSG mice carrying a human HLA-A2 transgene and found that this modification resulted in immune-dominant peptide specificities against EBV antigens. Recently a study by White et. al. indicated that results obtained in EBV infected humanized mice can be translated to human infections as they showed that infection of humanized mice with an EBNA3B deficient EBV displayed a strong resemblance to the outcome in human patients. In conclusion, humanized mice provide a representative model for EBV infection.

Although most studies have focused on viral infections in humanized mice, recently several groups reported the use of humanized mice for studies with Salmonella typhi. Infection with these bacteria gave rise to an immune pathology that resembled human typhoid fever. Of interest, the background of the humanized mouse made a difference in the outcome of infection; for humanized NSG mice the infection was lethal, whereas for humanized BRG mice it was not.

In summary, the recently developed humanized mouse models are a welcome addition to the field of infection biology. Even though they may not be able to fully resemble the human immune response at present, the current data is already promising and gives good reason to explore the use of these models for other infections.
2 SCOPE OF THE THESIS

To set up a humanized mouse model in which human immune cells develop de novo and use this model to study different aspects of human immunity.

2.1 SPECIFIC AIMS

**Paper 1:** Despite immunosuppressive treatments, graft rejection is still a major problem in the field of tissue transplantation. We used humanized mice to determine if this model could be used to study rejection of transplanted human allogenic β-islets, the treatment to overcome type I diabetes, *in vivo.*

**Paper 2:** Studies on differentiation of human immune cells *in vivo* are still few. Here we used humanized mice and hematopoietic stem cell transplant (HSCT) recipients to confirm newly found differentiation steps in CD56\textsuperscript{dim} NK cells *in vivo.*

**Paper 3:** Although various animal models are available for studies on mycobacterial infections, key elements of the human immune response are missing. To overcome these limitations we used humanized mice as a model to study human immune responses to mycobacterial infections.

**Paper 4:** Previous studies have validated the use of humanized mice for studies on EBV immunobiology. Based on these findings, we used humanized mice to get better insights in EBV induced latency.
3 RESULTS AND DISCUSSION

3.1 DEVELOPMENT OF HUMANIZED MICE

Throughout this thesis different approaches were used to generate humanized mice for our studies. These HIS mice were generated according to the original protocol by Traggiai et. al. in which new-born recipient mice are transplanted with human CD34+ cells after sub-lethal irradiation\textsuperscript{161}. This protocol is illustrated in figure 2. In papers I and II BRG mice were used, whereas in papers III and IV NSG mice were the recipient strain. While both mice lack mature T, B and NK cells we observed that use of the latter strain resulted in better and more stable engraftment in comparison to BRG mice even when we treated the pregnant dams with the myeloablative compound busulfan to precondition the new-borns (Figure S1, paper III). These results are in line with data presented by others\textsuperscript{87,105}.

3.2 PAPER I

In this paper we assessed whether humanized mice could be used for studies on human allograft rejection \textit{in vivo}. We generated humanized mice by transplanting CB derived CD34\textsuperscript{+} cells in new-born BRG mice after preconditioning with busulfan in utero and irradiation prior to transplantation. Before assessing transplant rejection \textit{in vivo}, we first determined the reactivity of human T cells derived from engrafted mice in a mixed lymphocyte reaction (MLR) to autologous and allogeneic dendritic cells. As was shown by others, reactivity to autologous mouse and human cells was minimal, whereas responses to allogeneic cells were detected (Figure 1).

Transplantation of pancreatic islets is used as a method to restore the pancreatic endocrine function in patients suffering from type I diabetes (T1D). Despite immunosuppressive measures grafted islets are subject to rejection over time\textsuperscript{167}. We reasoned that humanized mice could be an appropriate model to study this rejection in detail and if so could be used to improve current treatments. In order to assess graft rejection \textit{in vivo}, we transplanted human allogeneic pancreatic islets under the kidney capsule of humanized mice. Histological analysis of kidney sections containing the transplant and stained for insulin at 14 and 35 days after transplantation showed no
signs of rejection compared to non-humanized BRG controls. Furthermore, after staining with anti human CD3 antibodies we did not observe any infiltration of human CD3$^+$ T cells in the graft (Figure 2). To confirm these findings further, we evaluated the levels of C-peptide in the serum of transplanted mice. This method is commonly used in human recipients of islet grafts and is based on the fact that pro-insulin is cleaved into insulin and C-peptide$^{168}$. In line with the histology results we did not find differences in C-peptide levels between humanized and non-humanized mice (Table 1). Thus despite the promising ex vivo results, we were not able to establish humanized mice as a model to study rejection of human allografts.

While our results suggest that the HIS model is not suitable for rejection studies, work by Brehm et. al. showed that rejection of human β-cells was possible in humanized NOD/Rag1$^{-/-}$/IL2γ$^{-/-}$/Ins2$^{Akita}$ mice$^{169}$. These mice spontaneously develop hyperglycemia, which can be restored to normoglycemia by transplantation of mouse or human islets. When these mice were engrafted with human CD34$^+$ cells as newborns and transplanted with allogeneic human islets as adults the graft was rejected in 60% of the mice and mice became hyperglycemic again.

These findings imply that the reason why we did not observe rejection lies in the HIS model we used. In our model the frequency of human cells in the spleen was lower and less consistent than that observed by Brehm et. al. (38.8 ± 29.7% in our study vs 50.98 ± 4.9%). A study by Kirkiles-Smith et al. on rejection of human skin in SCID-beige mice transplanted with human CD34$^+$ cells showed that poorly reconstituted animals did not reject skin$^{170}$. Based on these findings the lower reconstitution can be thought of as one reason for the failure of graft rejection in our study. An other possible explanation for the lack of rejection could be the lower number of islets that we transplanted (300 or 500) in comparison to Brehm et. al. (4000), thereby providing less allo-antigens to induce rejection. Thus, even though our studies were not showing signs of allograft rejection, it should be possible to perform such studies after further optimization of the model.
NK cells are commonly divided into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells, where the former subset is the less mature and the latter is believed to be terminally differentiated \textsuperscript{12}. The recent findings on NK cell education and responses similar to those of the adaptive immune system, like expansion and contraction of a specific subset and the formation of memory cells \textsuperscript{171}, in response to in cytomegalovirus (CMV) infections prompted us to investigate whether CD56\textsuperscript{dim} cells could undergo further differentiation.

We found that human CD56\textsuperscript{dim} cells did indeed differentiate further. During this process the number of KIRs increased, the expression of the inhibitory receptor NKG2A was lost and the expression of CD57 increased. Although all these changes were found to be independently associated with reduced proliferative capacity in response to cytokine stimulation, we found that in NK cells expressing CD57, a marker that on T cells has been found to be associated with replicative senescence, proliferation in response to cytokines was virtually absent (figure 1 and 2).

Since CD56\textsuperscript{dim} cells seemed to acquire KIRs and CD57 in parallel, we wanted to determine if NK-cell education and the acquisition of self-KIRs was associated with acquisition of CD57. We found similar CD57 expression on educated and non-educated NK cells, indicating that the acquisition of these molecules was a parallel yet uncoupled event. In line with these data, we found that CD57 positive cells were less responsive to cytokine stimulation compared to CD57 negative cells, whereas no such difference was observed between educated and non-educated cells (figure 5 and 6).

In order to strengthen our findings with \textit{in vivo} data we studied NK cell reconstitution in humanized mice as well as in humans after hematopoietic stem cell transplantation (HSCT). In order to drive differentiation of NK cells in humanized BRG mice we made use of the previously reported finding that weekly treatment with trans-presented human IL-15 increased the frequency of NK cells and induced differentiation from CD56\textsuperscript{bright} to CD56\textsuperscript{dim} cells \textsuperscript{132}. Mice were analysed for expression of CD57 after 1 and 4 weeks of treatment. CD57 positive cells appeared after 1 week and further increased after 4 weeks in the spleen, liver and blood but not in the bone marrow or thymus, even though the total number of NK cells increased in all these organs. In line with our previous data, CD57 expression on NK cells mice was associated with the acquisition of KIRs and lack of proliferative capacity. Interestingly, NK cells in HSCT recipients also showed a gradual appearance of KIR\textsuperscript{+} and CD57\textsuperscript{+}
cells over time whereas these cells were not present in the first weeks after transplantation (figure 3 and 4).

To further confirm that CD57⁺KIR⁺ NK cells were in a late stage of differentiation we transferred sorted CD57⁺KIR⁻ or CD57⁺KIR⁺ cells into non-reconstituted BRG mice, treated them with trans-presented IL-15, and analysed the expression of both markers one week after transfer. We found that CD57⁺KIR⁺ cells had retained the same phenotype, whereas CD57⁺KIR⁻ cells had started to increase the expression of CD57 and, albeit to a lesser extend that of KIRs (figure 3).

Our findings in this paper allow for further dissection of NK cell differentiation. Other differentiation markers have also been proposed to further delineate the CD56dim NK cell subset 172-175. It remains to be investigated if the findings of these studies can be unified into one model. Of relevance for such studies is our observation that, for the markers we investigated, differentiation of NK in humanized mice closely resembles that observed in humans.

3.4 PAPER III

While humanized mice are well established as a model for pathogens only infecting human immune cells, their use for pathogens that have a broader host tropism has not been explored very much. We investigated if humanized mice could be used to study human immune responses to mycobacterial disease.

In order to investigate the human immune response to mycobacterial infections we infected humanized NSG mice with 1x10⁶ M. bovis BCG i.v. or 250 M. tuberculosis bacilli via aerosol infection. After 4 weeks we found that frequencies of human CD4⁻ T cells in the spleen and CD3⁺ T cells in the liver and lung were increased compared to non-humanized infected NSG mice. Both CD4⁺ and CD8⁺ T cells showed a shift from a naïve CD45RA⁺CCR7⁺ phenotype to a CD45RA⁻CCR7⁻ effector memory phenotype and more IFN-γ and TNF-α positive cells were detected after stimulation with mitogen. Corroborating this data we found that mRNA transcript levels of IFN-γ and the IFN-γ regulated T cell chemotactic chemokines CXCL9 and CXCL10 were significantly higher in lungs and livers of infected mice (Figure 1, 2, 5).

Messenger RNA levels of the macrophage tactic chemokine CCL2 were also slightly higher in infected animals and lungs and livers of infected mice did indeed contain increased frequencies of human myeloid cells in lungs and livers (Figure 5). These findings suggested that the infection had elicited a strong immune response.
Therefore it was surprising to find that BCG titers were higher in lungs and livers of humanized mice compared to the non-humanized controls (Figure 3).

The higher bacterial burden in humanized mice indicated that the presence of human immune cells had a detrimental effect on the bacterial control. Since macrophages are the primary target cell for mycobacteria we investigated if human macrophages were more susceptible to infection by BCG and if these bacteria replicated more in human macrophages compared to mouse macrophages. After incubation of human and mouse macrophages with BCG we found slightly more bacteria associated to human cells, but no difference in the growth rate was observed in the following days (figure S4). Whether elimination of mycobacteria by human macrophages was impaired in vivo was addressed next by determining mRNA transcripts of the IFN-γ inducible enzymes GP91phox (a component of NADPH oxidase) and iNOS, both of which have been found to be important in control of M. tuberculosis infection \(^{176,177}\). No changes in transcript levels were found in response to infection, suggesting that this part of the immune response to TB infection was not functional in humanized mice (figure S4).

Although T cells should have a protective role in TB infection, we also investigated if they contributed to the increased bacterial burdens in humanized mice. We hypothesized that even though they showed an activated phenotype after infection, T cells might have functional impairments. Therefore we analysed the expression of the Programmed Death-1 (PD-1) and CD57 molecules on T cells in infected and control mice. Signalling through PD-1 results in down-regulation of T cell function \(^{178}\). As discussed in paper II, CD57 is a marker for replicative senescence in T cells and NK cells \(^{179}\). We found that the frequency PD-1 and CD57 expressing cells was greatly increased after infection, indicating that even though the T cells had an activated phenotype, their function was impaired (figure 2 and S3). In line with these findings, Brainard et. al. have described that high levels of PD-1 expression in HIV infected humanized mice correlated with increased viral titers \(^{180}\).

Based on these findings we decided to study the role of T cells in the control of BCG infection by depleting mice of CD4\(^+\) or CD8\(^+\) T cells via inoculation of specific antibodies. While depletion of CD8\(^+\) cells did not have any effect, depletion of CD4\(^+\) cells paradoxically resulted in reduced bacterial numbers compared to non-depleted control mice (figure 3).
Despite the lack of bacterial control, histological analysis of lungs and livers from BCG-infected mice showed granulomas similar to those found in human TB, whereas such lesions were absent in non-humanized infected mice (figure 4 and 6). The granulomas were well defined and consisted of a core with mainly human CD68 positive macrophages, epithelioid cells and occasional multinucleated giant cells. CD3$^+$ lymphocytes and a fibrotic capsule surrounded the core. Notably, mycobacteria preferentially located in the macrophage core of the granuloma (figure 4 and S6).

*M. tuberculosis* infection of humanized mice and non humanized mice gave rise to macroscopically visible granulomas in liver and lungs (figure 6). Although it has been shown that granuloma like structures can develop in mice devoid of T cells $^{181,182}$ we were surprised to find lesions that looked similar upon macroscopic examination in the lungs of controls. However, histological examination of livers and lungs showed that the pathology induced by the infection was more severe in livers from humanized mice (figure 6). Similar to the BCG induced granulomas, CD3$^+$ cells located in the periphery of the granuloma and multinucleated giant cells were only detected in humanized mice (figure S6). Central necrosis was observed in lung granulomas of both humanized and non-humanized mice, in the former the extent of necrosis was strikingly bigger, suggesting that this feature of human TB granulomas was supported by human cells (figure 6). Further studies are required to shed light on this observation though.

Reactivation of TB in individuals that have contracted HIV is believed to be a result of break down of granulomas due to a reduction in CD4$^+$ T cells $^{47}$. By using mice deficient in T cell subpopulations we found that CD4$^+$ but not CD8$^+$ cells were indeed essential for formation and/or maintenance of granulomas in BCG-infected humanized mice (figure 3 and 5). Together with the finding that CD4 depletion resulted in a reduction of bacteria, our data is supporting the hypothesis that granulomas are forming a safe environment in which bacteria can replicate relatively unharmed $^{49,50}$.

Further developments in recipient mouse strains would likely aid in improving the cellular immune response to the infection. Given the fact that mycobacteria are not restricted to infection of human cells, improving the human myeloid compartment $^{126,130}$ or improving antigen presentation by mouse myeloid cells through the use of NSG mice expressing human HLA molecules $^{154,183}$ would be a step forward to a complete model for human mycobacterial infections. Despite these limitations, our work demonstrates that HIS mice can be used to study components of the human immune response to mycobacterial infections, specifically the formation of granulomas.
Several studies have illustrated the viability of HIS mice for studies on the immunobiology of EBV, which cannot infect other than human cells. In paper IV we evaluated the possibilities to use humanized mice as a model to study EBV latency.

EBV preferably infects B cells and CD21 (complement receptor 2) is essential for the infection. FACS analysis of human B cells derived from blood and spleens of humanized mice showed that, in comparison to human cord blood B cells, CD21 expression is lower. Despite the lower expression of the receptor for EBV, ex vivo incubation of splenocytes from humanized mice resulted in B cell transformation, indicating that in agreement with others, B cells from humanized mice can be infected by EBV (figure S1).

In our in vivo studies, we inoculated 8 to 10 weeks old humanized mice with EBV i.p. and evaluated the outcome of infection 4 weeks later. Similar to data reported by others, we found an expansion of the CD8+ T cell compartment (figure 1 and 2). Although most of the inoculated mice were confirmed to contain EBV transcripts (Table 1), a proportion of these mice showed B cell tumors in the spleen whereas other mice seemed to control the infection (Figure 1). In line with this, splenocytes proliferated after ex vivo culture only in a proportion of the mice (table 2 and figure 2). Thus, in our hands the outcome of infection with EBV proved to be highly variable.

Previous reports showed a critical role for T cells in the control of EBV infection in vivo, as evaluated by the appearance or increase of tumors and viral particles. We evaluated whether T cells could modulate the ex vivo growth of infected cells. Surprisingly, we found that inhibition of T cell function by addition of cyclosporine A (CsA), led to reduced proliferation of infected cells compared to parallel cultures without CsA (figure 3). FACS analysis of both cultures showed that over 97% of the cells in the culture were CD19 positive B cells, thus eliminating the possibility that T cell proliferation could account for the higher number of proliferating cells in absence of CsA. Thus, it appeared that T cells provided substantial support to EBV transformed B cells. As type III latency cells spontaneously proliferate in culture we speculated that T cells supported proliferation of non-type III cells.
To confirm the presence of such latency I/II cells we determined which viral promoters were used by RT-PCR. Here we found that both C-promoter and Q-promoter transcripts were present in spleens from infected mice, respectively confirming the presence of type III and non-type III latency (table 1).

In order to further evaluate which T cells were responsible for the growth stimulation *ex vivo*, we depleted infected mice *in vivo* of either CD4⁺ or CD8⁺ cells. Whereas CD8 depletion increased the occurrence of tumors and resulted in increased proliferation *ex vivo* (table 2 and figure 2), no tumors or *ex vivo* outgrowing cells were found in CD4⁺ cell depleted mice. The proliferation inhibiting effect of CsA was greater in splenocytes from CD8⁺ cell-depleted mice (figure 3), suggesting that CD4⁺ cells were responsible for the transition to and/or proliferation of type I/II latency cells. In line with this, transcript levels for the EBV Q-promoter (Qp), which is associated to type I and II latency, were higher in the majority of CD8⁺ cell-depleted mice compared to non-depleted littermate controls (figure 4). The lack of Qp transcripts in 3 out of 4 CD4⁺ depleted mice further supported our hypothesis that CD4⁺ cells were promoting the generation and/or survival of non-type III latency (table 1).

In line with our data, we recently found that soluble factors produced by CD4⁺ cells modulate EBV latency *in vitro* ⁶⁹. Although other *in vitro* studies have shown that several CD4⁺ T cell derived cytokines can support the transition to and growth of non-type III latency cells ⁶⁶⁻⁶⁸, RT-PCR analysis of IL-4, -10 and -21 did not show any correlation with Qp usage in splenocytes of humanized mice (data not shown).

Taken together, the results in this manuscript confirm reports by others that different forms of EBV latency are present in humanized mice ¹⁵⁵,¹⁵⁶. Our findings on the role of CD4⁺ T cells in EBV latency illustrate that humanized mice can provide valuable information about this process *in vivo*. 
4 CONCLUSIONS

The work presented in this thesis provides further insight in the possibilities and limitations that currently available humanized mouse models. We confirmed that HIS mice could be used for studies on human immune cell development and immune responses to different pathogens. The key findings of the papers constituting this thesis are:

- Human T cells from humanized mice are functional \textit{in vitro} but do not reject allogeneic β-islets after transplantation \textit{in vivo} (paper I).
- NK cells in humanized mice and HSCT patients follow the same differentiation pattern and both acquire CD57 and KIRs in a late stage of differentiation (paper II).
- Humanized mice infected with mycobacteria develop granulomas resembling those found in tuberculosis patients, but the human transplant does not protect the mice from the infection. The presence and features of the granulomas was completely dependent on the human transplant and in the case of BCG-infection on the activity of CD4$^+$ cells (paper III).
- Different forms of EBV latency are present in EBV infected humanized mice. CD4$^+$ cells promote the induction and/or maintenance of non-type III latency (paper IV).

Notwithstanding the promising results we obtained, our studies faced several limitations. Notably, the BRG and NSG mice we used did not allow for proper T cell selection. Although T cell activation was detected in response to both mycobacterial and EBV infections, we could not detect antigen-specific cells in either study. As shown by others, the use of HLA class I and II transgenic recipient mice would be a major step forward in studies like ours. As mice with both transgenes have recently been developed\textsuperscript{154,159}, the technical limitations to validate our results in a setting were T cells are properly educated have been eliminated.

Another aspect that should not be ignored is the residual mouse immune system and reactivity and cross reactivity, or the lack thereof, of mouse and human cytokines and growth factors produced by both hematopoietic and stromal cells, which are required for proper function of the immune system. The lack of cross reactivity between the
human transplant and mouse recipient could be both at the level of the hematopoietic cell development but also on their function due to inflammatory cytokines or chemokines.

Given the rapid and ongoing advances in this field I do not doubt that the limitations above will be addressed and turn humanized mouse models into more powerful models than they already are. Parallel to the development of new recipient mouse strains, advances have been made in the generation of hematopoietic stem cells from inducible pluripotent stem cells. In the future, selection of appropriate recipient mice and in vitro manipulation of HSCs could therefore allow for the generation of ‘tailor made’ humanized mice. I therefore do not expect that there will be one general humanized mouse model. Rather, using the currently available tools, mice specific for the question that is being addressed can be generated.

In conclusion, our results contribute to a rapidly expanding field and illustrate that humanized mice have a strong potential for use in a variety of applications.
ACKNOWLEDGEMENTS

Along the long and winding road that led up to the writing of this thesis I have met many people who, every one in his or her own way, have contributed to this work and through their support have prevented me from turning into a murinized human 😆.

First and foremost I want to thank my supervisor Martin Rottenberg, for accepting me as a PhD student in what turned out to be a very intricate and diverse PhD project. Despite all the challenges that were thrown at me by the mice you always kept me motivated and pushed me to keep trying.

My co-supervisors: Noemi Nagy, for teaching me many things on EBV and for all the hard work trying to get the most out of every experiment and for a large variety of ice cream. Hans Wigzell for the never-ending stream of knowledge and willingness to share it with me whenever you had time. Marianne Jansson for taking the time to listen to my results and all your suggestions.

Small Berit, who was always behind me (or was it your sister?) 😊. While your Olympic adventure was the start of my Stockholm adventure I’m happy that you came back so that we could design our super secret experiments together.

Big Berit for always being kind and for withstanding all the things that for some reason only happened to you and I denied knowing anything about.

Suman for teaching me how to read the clock the Indian way.

Eni, thank you for stopping to use your karate skills on me.

Other past and present members of the group for the great atmosphere and all the fun: Carina, Ahmed, Yeh, Kiran, Ann-Kathrin, Christian and Hilda.

All the students that I made to calculate by head in the lab: Christiné, Frieda, Steffi, Cindy, Patrik, Fanny and Steve.

The staff of the MTC and Astrid Fagraeus animal facilities. Without your help it would have been impossible to perform my studies. Thanks for taking care of my lovely little mice.

All the nurses from the Karolinska hospitals in Solna and Huddinge who helped in collecting cord blood, which was so vital for my work.

All co-authors of the papers in this thesis and in particular the people with whom I have worked closely: Stella and Malin for the help in setting up the model, Niklas, Sandra and Kalle for the devotion on the NK cell work. Eva Klein for the endless stream of new ideas and members of your group: Lori, Dani, Eahsan, Harsha, Dorina and Mónika for the in vivo and in vitro work on EBV.
My mentor Mikael Rhen and the present and former members of his group: Spe, Aurel, Naeem, Sem, Syed, Cesar, Carlos, Chriselle, Sarah, and Sabrina for your company and all the cakes, cookies, chocolate, sweets and liquorice.

Members of the various journal clubs that I have participated in over the years: Markus, Lech, Jola, Emma, Rozina, Lotta, Chaniya, Rebecca, Isabelle, Tony, Vishnu, Susanne, Rashid and Katja for all the stimulating discussions.

Francesca Chiodi and the members of her group for sharing reagents and discussions on human cells.

Julius and Kanth for all the fun during our big and early experiments.

Isabel and Romanico for exploring in vivo imaging together

All other people in MTC and KI who I’ve met along the way. The list is simply too long to put here.

My neighbours in Lappis, particularly Sagar for all the things we shouldn’t have done to others but would do again should we get the chance, and Fi, for your never disappearing smile.

Members of the Stockholm Australian Football Federation for good times and for giving me something to focus on at the football field.

Everyone that has given me support from abroad: particularly Joep, Iva, Ana, Jürgen, Anna and Margaret.

Mijn ouders. Bedankt voor de vrijheid en steun die jullie me altijd hebben gegeven tijdens de reis die ik ben begonnen om mijn ambities waar te maken en me via Utrecht en Melbourne naar Stockholm heeft gebracht, ver van jullie vandaan. Jullie zijn altijd in me blijven geloven ook al werd het steeds moeilijker of precies te begrijpen waar ik nu eigenlijk aan werkte.

Marta, far away yet always close…..
6 REFERENCES


