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NATURAL KILLER CELL EDUCATION UNDER CONDITIONS OF PERTURBED SENSING OF MHC - STUDIES IN MHC-I MOSAIC AND CHIMAERIC MICE

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Karolinska
Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by E-Print AB 2017
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ISBN 978-91-7676-727-6
Cover photo by SS

NATURAL KILLER CELL EDUCATION UNDER CONDITIONS OF PERTURBED SENSING OF MHC - STUDIES IN MHC-I MOSAIC AND CHIMAERIC MICE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen wid Karolinska Institutet offentligen försvaras I Föreläsningssalen Hillarp, Retziusväg 8, Karolinska Institutet, Solna

Tisdagen den 13 juni, 2017, kl 9:30

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To the loving memory of my grandparents

and to my family

ABSTRACT

Natural killer (NK) cells are innate lymphocytes that play a role in infections, tumours and transplantation. Their activating and inhibitory receptors sense cell surface ligands on potential target cells. Increased levels of stress induced activating ligands and decreased levels of inhibitory MHC class I (MHC-I) ligands cause activation of NK cells. NK cells are “educated” to tolerate self, and the “rheostat model” proposes that this is a reversible and quantitative process, where the degree of responsiveness acquired by each NK cell is proportional to the amount of perceived inhibitory MHC-I signals. Assuming that the NK cell integrates MHC-I input over multiple cell encounters, education may also depend on the ratio between cells with and without a critical MHC- I ligand, e.g. in chimaerism after haematopoietic transplantation. This thesis addressed how NK cell education is affected by perturbed sensing of MHC-I, and how the process is influenced quantitatively by the frequency of cells with a given MHC-I ligand. “Allele specific” as well as “general” missing self reactivity (targets lacking one or all host MHC-I molecules respectively), against normal and tumour target cells, was analysed.

The DL6 mouse strain presents a mosaic expression of an MHC-I transgene such that each cell either expresses it ($D^{d+)}$ or not (D^{d-}). To test NK cell killing *in vivo* of two different targets in the presence of a control cell population in these mice, one aim was to develop an assay to measure rejection of three fluorochrome labelled targets in parallel. Using this assay, all mosaic DL6 mice were completely tolerant towards normal D^{d-} spleen cells (“allele specific” missing self), even if they had a relatively high proportion of D^{d+} vs. D^{d-} cells. There were varying degrees of impaired reactivity in the mosaic mice upon challenge with MHC-I defective spleen cells (“general” missing self), with a moderate inverse correlation between the number of host D^{d+} cells and the survival of these target cells. Tolerance towards D^{d-} RMA tumour cells representing “allele specific” missing self was not complete; partial rejection was observed in many DL6 mice. There was a weak inverse correlation between the number of D^{d+} cells and the survival of these target cells; in particular, there was an efficient rejection in all mice with >83% D^{d+} cells. The frequency of D^{d+} cells did not influence rejection of MHC-I deficient RMA-S lymphoma cells; all DL6 mice rejected these cells efficiently. In chimaera studies where D^{d+} bone marrow was used to reconstitute mosaic DL6 mice, there was again complete tolerance towards normal D^{d-} spleen cells, indicating that a fraction of ligand deficient non-haematopoietic cells is sufficient to induce tolerance to normal cells of this phenotype. Again, the results differed when tumour cells were used; there was significant (but reduced) capacity for “allele specific” missing self rejection in these chimaeras. In the reverse setting, mixed bone marrow chimaeras were constructed in which different ratios of D^{d+} and D^{d-} cells were transplanted to mice with normal D^{d} expression in the non-haematopoietic compartment. Significant but reduced capacity for rejection of D^{d-} RMA tumour cells was observed, despite apparent tolerance towards normal D^{d-} cells. Finally, when MHC-I sensing by NK cells in B6 mice was perturbed by treatment with antibodies against inhibitory Ly49I/C receptors, it led to impairment of “general” missing self reactivity towards MHC-I deficient spleen cells, despite increased killing of MHC-I expressing RMA tumour cells. Both changes occurred within 24 hours after inhibitory receptor blockade, and it was not possible to separate them in kinetic studies. As in the DL6 mice, efficient killing of MHC-I defective RMA-S cells and tolerance towards normal MHC-I expressing cells, were preserved.

The results show that tolerance develops robustly towards normal host cells lacking one MHC-I ligand, i.e. the D^{d-} cells are critical and dominant tutors in the education process even if they are in minority. A model is proposed in which the education sets responsiveness for each NK cell such that

tolerance is ensured towards all normal cells in the host. This occurs via multiple cell encounters, and results in a reduction of responsiveness in certain NK cell subsets with inhibitory receptors for D^d molecules, correlating with the proportion of D^{d-} vs. D^{d+} cells in the environment. This D^{d+} cell frequency dependent, quantitative educational effect is revealed when using MHC-I deficient spleen cells or D^{d-} tumour cells as targets. A similar interpretation is offered for the results observed in antibody mediated inhibitory receptor blockade, where the perturbed MHC-I sensing is manifested as a reduced inhibitory signal from all cells to certain NK cell subsets, rather than complete lack of inhibitory signal from a fraction of the host cells. The results are discussed in the context of testable experimental predictions of *in vitro* NK cell responsiveness in mice with mosaic or chimaeric MHC-I expression, as well as in the context of implications for clinical immunotherapy against cancer.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell mediated cytotoxicity
AML	Acute myeloid leukaemia
β_2m	β_2 microglobulin
BM	Bone marrow
CAR	Chimaeric antigen receptors
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
DNAM-1	DNAX accessory molecule-1
DAP-10, 12	DNAX activating protein of 10 or 12 kD
GVHD	Graft versus host disease
GvL	Graft versus leukaemia
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplantation
IFN-γ	Interferon γ
IL	Interleukin
ILCs	Innate lymphoid cells
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KLRG1	Killer cell lectin like receptor G1
KIR	Killer immunoglobulin like receptors
Ly49r	Ly49 receptors
MCMV	Mouse cytomegalovirus
MHC	Major histocompatibility complex
mNK	Mature NK cells
NK	Natural killer cells
TNF-α	Tumour necrosis factor α
TRAIL	Tumour necrosis factor (TNF) related apoptosis inducing ligand

1 INTRODUCTION

1.1 BACKGROUND

The immune system is a defence system composed of a network of cells, substances and tissues which coordinate to protect the host from foreign invading agents or internal anomalous changes. Its essential features include mechanisms to detect foreign or altered components, to communicate within and among different arms of the system and to elicit responses by various fractions of the system. Conventionally, the immune system in higher vertebrates has been divided into the non-specific innate section and the more specific adaptive section. The innate immune system constitutes the first line of defence and comprises mostly non-specific mechanisms e.g. mast cells, phagocytes e.g. granulocytes (neutrophils), macrophages and dendritic cells, eosinophils, basophils, natural killer lymphocytes, complement system, antimicrobial peptides, anatomical barriers, etc. Pattern recognition receptors are also included in these, of which, Toll like receptor family is an important type in recognizing pathogen-associated molecular patterns. The adaptive immune system forms the second and more efficient line of defence as it retains memory of previous antigen encounters. It consists of T lymphocytes, B lymphocytes and antigen presenting cells e.g. dendritic cells, macrophages, etc. However, with advances in research, considerable overlap and cross-talk between the two types of immune systems is being seen, thus the division is seen as more labile. The studies presented in this thesis pertain to mouse NK cells and, therefore, general characteristics of mouse NK cells are described in more detail in the following sections, while human NK cells are mentioned briefly for comparison, and sometimes in detail when there are interesting differences between the species (Table 1).

Table 1. A comparison of human and mouse NK cells

NK cell properties	Human	Mouse
Frequencies	5-15% of PBMCs	2-5% of spleen lymphocytes
Markers used for identification	$CD3^-CD56^+$	CD3-NK1.1$^+$/DX5$^+$/NKp46$^+$
Major inhibitory receptors for MHC-I	KIR family	Ly49 receptor family
Inhibitory receptors for HLA-E/Qa1-b	CD94/NKG2A	CD94/NKG2A
Expression pattern of inhibitory receptors	Variegated	Variegated
Inhibitory signalling motifs	ITIMs in KIR family (two in each monomer)	ITIMs in Ly49 receptor family (two in each homodimer)
Activating receptors (examples)	NCR (NKp46, NKp30,NKp44) NKG2D, activating KIRs, CD16	NCR (NKp46), NKG2D, Ly49D, Ly49H, and CD16

1.2 NATURAL KILLER CELLS

1.2.1 General background and biology

Natural killer (NK) cells (reviewed in [1]) are important cells of the immune system. Although mostly categorized within the innate component, there are studies showing their role in the adaptive immune component, and as bridges between the two systems [2, 3]. NK cells are generally conserved across species albeit with changes in surface proteins [4]. They play an important role in controlling viral infections and in anti-tumour surveillance and responses. They also partake in pregnancy and graft rejection (including graft versus tumour effects).

NK cell development is independent of thymus and gene rearrangement in their receptors, which are germ line encoded. As components of innate immune system, they bypass the need for prior stimulation, unlike T and B lymphocytes, and elicit effector functions upon primary exposure to the target, spontaneously mounting killing responses to targets and thus, earning the title 'Natural Killers'.

NK cells communicate with surrounding cells in several ways. It could be via soluble substances, which act on cells independent of cell-cell contact. Upon contact with a target cell, they may form an immunologic synapse. After moving away from target cells, they may also maintain contact with them at distances via nanotubes [5]. Different pathways are set off as a result of these interactions. NK cells are capable of producing lymphokines, mainly interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and causing cytokine induced cell damage. They also show direct cytolytic activity via release of cytotoxic granule contents, i.e. perforins and granzymes in target cell proximity, which leads to its apoptosis. Another mechanism used is Fas-Fas ligand induced apoptosis (Figure 1A) [6]. With respect to these effector mechanisms of cytotoxicity, NK cells share many features with cytotoxic T cells [7]. Major differences between these two cell types are a) T cells must mature and undergo quality control in the thymus, b) T cells require antigen specific activation and clonal expansion in lymphoid organs, before they can become cytolytic effector cells. Some NK cells may pass through the thymus, but most mature independently of the thymus. Furthermore, NK cells are present as cytotoxic effector cells in blood and tissues. They may need regular contacts with dendritic cells to remain primed for cytotoxic action, but they do not require proliferation and clonal expansion. The two effector cells, therefore, engage with different kinetics during immune responses (Figure 1B) [8]. NK cells act immediately and their number as well cytotoxic function can be augmented within 1-3 days. Cytotoxic T cells require 4-5 days before they can act [9]. NK cells may interact with other components of the immune system in a regulatory capacity as well [1, 3, 10].

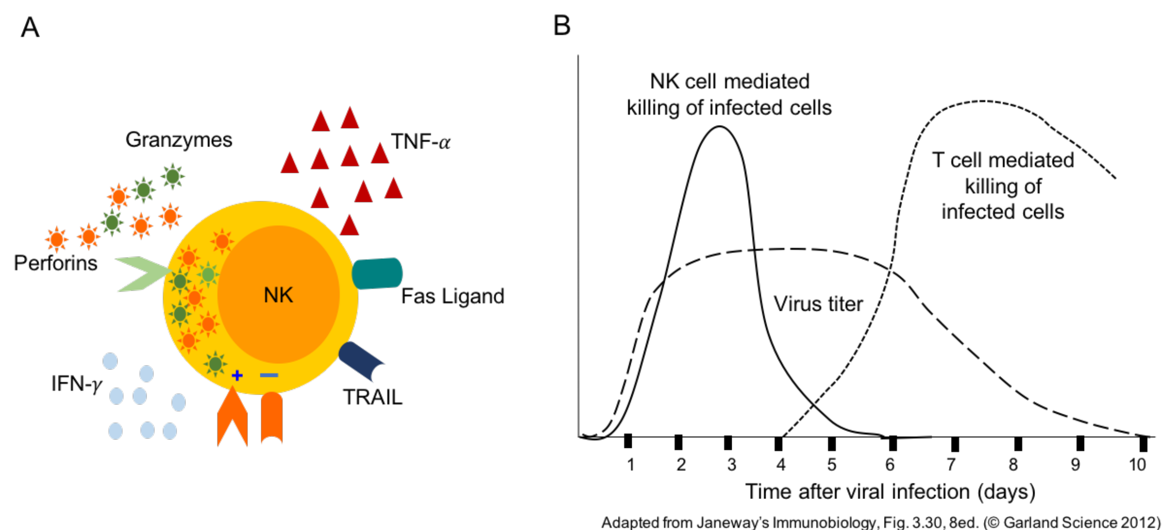


Figure 1. General NK cell properties. A: Diagrammatic presentation of some central NK cell effector functions. B: Schematic presentation of kinetics of NK cell and T cell responses during a viral infection.

Several other innate lymphocytes (ILCs) have been discovered recently, defined as a group of lymphocytes lacking receptor gene rearrangement. Mostly tissue-residents, they do not form a major part of the peripheral blood lymphocyte population. ILCs are activated by cytokines and activating receptors and take part in early responses in infection, allergy, autoimmunity as well as in tissue repair. Due to their interactions with other immune cells, especially via cytokines, they also have an immunomodulatory role [11]. Based on development, phenotype and function, three main groups of ILCs have been identified. NK cells belong to group 1 ILCs and share certain features with other ILC1s, especially regarding roles in inflammation and infection, but they are developmentally distinct from each other [12].

1.2.2 Morphology, distribution and cell surface phenotype

Morphologically, NK cells have a high nucleus to cytoplasm ratio and other features making them indistinguishable from other lymphocytes. They may, however, have large azurophilic granules in their cytoplasm, which contain cytotoxic substances [13], hence, the nomenclature by Loughran in 1985 i.e. large granular lymphocytes.

NK cells are mostly found in peripheral blood, lymphoid organs and to some extent in bone marrow. In physiological conditions, they have been seen to locate to medulla and paracortex of lymphoid tissues but may move to maintain contact with dendritic cells or come in proximity with T cells [14]. In humans, circulating peripheral blood mononuclear cells contain 5-15% NK cells. In mice, NK cells are usually isolated from spleens where they compose 2-5% of the lymphocytes. Several tissue resident NK cell populations have been identified and although they share properties with circulating NK cells (of the bone marrow (BM) origin), they may represent NK cells from a different lineage or progenitor (for example liver NK cells) [15, 16].

Like other cells, NK cells are identified by surface expression of protein markers many of which are classified as cluster of differentiation (CD) markers. A general classification of NK cells in humans is $CD3^-CD56^+$ cells while in mice, $CD3^-NK1.1^+$, $CD3^-NKp46^+$ or $CD3^-DX5^+$ is used. NK cells are then further classified phenotypically into specific subsets or maturation stages, depending on the research question under investigation. The details of these markers are discussed in the following sections.

1.2.3 Development and maturation

Similar to the other innate lymphoid cells, NK cells originate from haematopoietic stem cell (HSC) precursors. The bone marrow is the main site of NK cell development in the adult mouse. The common lymphoid progenitor (CLP) commit to T/B cell or ILC lineage prior to antigen specific receptor gene recombination. Under the effect of transcription factors Nfil3 and TOX, the CLP passes to the ILC/NK cell precursor stage. From here, the expression of GATA3 and Id2 transcription factors mark its commitment as the ILC precursor, which gives rise to ILCs. Group 1 ILCs rely on the transcription factor T-bet for development and produce IFN-γ [12]. The ILC-2 group is characterized by interleukins (IL) -4, 5 and 13 production and dependence on GATA-3 and RORα transcription factors. Group 3 ILCs require transcription factor RORγt and produce IL-17 and 22 [11, 12]. From the ILC/NK cell precursor stage, a pre NK cell precursor develops, which under the effect of transcription factors Eomes, T-bet and Id2, passes through the NK cell precursor and immature NK cell stage, and later completes development of NK cells in the periphery. Transcription factors T-bet and Eomes provide two checkpoints for NK cell maturation, especially in the BM medulla [17, 18].

NK cells undergo several developmental stages in the bone marrow, defined by surface markers including CD122 (IL2Rβ), NK1.1 and DX5 [19]. These stages correlate not only with surface marker acquisition, e.g. receptors, but also with functional capacity. Together, both confer education and expansion to NK cell clones. The NK-committed progenitor called pre-NKP, arises from the CLP and lacks IL-2Rβ (CD122) [20]. The $CD122^+NK1.1^-$ NK cell precursors or NKP, previously thought of as the first NK-lineage committed precursor [21], give rise to $NK1.1^+$ $DX5^-$ immature NK (iNK) cells and $DX5^+$ $Ly49^+$ mature NK (mNK) cells [20]. These mNK effector cells are functionally competent and are capable of producing cytokines and causing cytotoxicity. mNK cells emigrate from the bone marrow and constitute the main NK cell population in the peripheral lymphoid organs e.g. spleen and lymph nodes [21]. Based on the density of CD27 (member of surface TNF receptor superfamily, binding CD70) and Mac-1 (CD11b integrin, adheres to extracellular matrix), four developmental subsets having different functional capacities, have been identified in the periphery [21], also considered as the four terminal maturation stages in NK development [22]. Upon activation, however, NK cells may change surface expression of markers [20].

NK cells also express a variety of surface markers, some of which are tightly associated with a function. Many of them mark the different developmental stages in NK cell lymphopoiesis. DX5 (CD49b, binds extracellular matrix) is a component of certain integrins and helps in cell migration not only during development and recirculation, but also in wound healing. CD69 is an early activation antigen. NK cells also secrete chemokines (e.g. CXL10, CCL3, etc.) and express several cytokine receptors (e.g. IL-2R, IL-12R, IL-15R, IL-18R, IL-21R, CXCR3,

CCR1, etc.) involved in their proliferation, activation, expansion, immune regulation and migration. Among them, IL-21 plays an important role in NK cell expansion [23-28]. IL-15 is specifically required for NK cell lymphopoiesis. IL-15 and interaction with dendritic cells (especially in lymph nodes) are important for their development, survival, activation and proliferation [29]. Other markers are associated with cytotoxicity e.g. granzymes, granulysin, CD107α, Fas ligand, TRAIL, etc. [29, 30].

In humans, based on the selective density of surface CD56 (an adhesion molecule) expression, two subsets have been identified. $CD56^{dim}$ NK cells constituting about 90%, are the major cytotoxic subset whereas $CD56^{bright}$, making up about 10%, are the major cytokine (e.g. IFN-γ) producers [30].

1.2.4 Role in physiology and pathology

Due to their multifaceted effector functions, NK cells play important roles in physiological conditions e.g. pregnancy, as well as in disease situations e.g. viral infections, tumour rejection, etc. More aspects of NK cells' functions are currently being investigated in research. A few physiological and pathological states are described below.

1.2.4.1 Role in pregnancy

NK cells play an important role under physiological conditions in pregnancy. Under hormonal influence during pregnancy, a population of peripheral blood $CD56^{bright}$ $CD16^{-}$ NK cells, referred to as uterine NK cells, is recruited to the materno-foetal interface. This is interesting as it is a site of semi-allogeneic interaction where NK effector cells should exhibit a missing-self response and eradicate foreign tissue. On the contrary, under physiological conditions, here they expand and take part in immunomodulation i.e. immunoregulation. They promote trophoblast and placental growth via angiogenesis without maternal immunosuppression or foetal rejection, and which may be attributed to the release of transforming growth factor- β (TGF-β). This is important for implantation and maintenance of pregnancy and abnormalities in this process lead to an unfavourable environment for the embryo/foetus in the uterus leading to abrogation of pregnancy. This is also another indicator of how NK cells adapt their responses depending on the environment and regulate immune environment [31-34]. Outcomes of pregnancy and foetal health parameters have been related to maternal KIR (Killer immunoglobulin like receptors, NK cell receptors in humans and described in detail later) expression. Paternal HLA (Human leukocyte antigen, major histocompatibility complex antigens class I (MHC-I)) has also been seen to play a role, in that, certain combinations of maternal KIR on uterine NK cells and paternal HLA molecules on trophoblasts, may lead to unfavourable outcomes in pregnancy e.g. recurrent spontaneous abortions, pre-eclampsia, intrauterine growth restriction, etc. [35].

1.2.4.2 Role in infections

NK cells play important roles in fighting infections, especially those caused by intracellular pathogens e.g. viruses, bacteria, protozoa (e.g. *Leishmania*), parasites (e.g. *Toxoplasma gondii*) [36]. Presence of NK cells in organs allows them to fight specific infections e.g. viral infections in the liver. Due to the nature of their responses, they might also participate in disease pathogenesis in addition to their anti-viral effects [37]. Investigation of NK cell

responses in context of many infections has shown that they are among the first responders and expand in response to e.g. MCMV (mouse cytomegalovirus), HCMV (human cytomegalovirus), Listeria monocytogenes, Herpes, Hepatitis B and C, Hanta virus, Chikungunya virus etc. [29, 38-41]. In HIV infection, NK cells help in infection control by killing infected cells [36].

Studies have shown that due to their memory-like and antigen-specific responses to viral infections and crosstalk with other immune cells e.g. dendritic cells, etc., their role in viral infections is complex and not limited to that of a classical innate immune cell [42]. Although NK cells have been considered as innate immune system components and thus unable to mount memory-based responses ascribed to the adaptive immune system, studies have shown that prior antigen activation plays a role in enhancing NK cell responses. For example, in the case of MCMV infection, Ly49H$^+$ NK cells antigen specific for MCMV protein m157, respond faster. Once activated, some of these cells remain as long-lived, highly responding cells, reminiscent of memory T cells [43]. Other memory-like responses by NK cells can be induced by cytokine stimulation without any known antigen dependency [44].

1.2.4.3 Role in autoimmunity

NK cells have been studied in a variety of autoimmune diseases and experimental models. The picture is very complex and there is evidence that NK cells can either promote or counteract autoimmunity, even within the same disease. This may depend on the phase of disease development [45]. NK cells may cause severe tissue damaging inflammations in response to viruses [40]. Some examples of autoimmune conditions where NK cells have been suggested to play a role in pathogenesis include Juvenile Rheumatoid Arthritis, Type 1 Diabetes, Autoimmune Thyroid diseases, etc. [46-49].

1.2.4.4 Role in transplant & tumour immunology

NK cells mediate bone marrow rejection even in irradiated in mice, and play a role in the pathophysiology of graft versus host disease (by preventing it). Recent studies have shown that they influence solid organ allografts as well [50]. As mentioned earlier, they play a vital role in killing tumours especially those of the haematopoietic and lymphoid system. Primary or transplanted tumours in mice grow better in NK cell depleted conditions [51]. The same has been observed in solid organ tumour studies, where several strategies are being investigated that may increase NK cell potential to kill cancer cells within the tumour microenvironment [52]. These roles of NK cells are discussed in more detail later.

1.3 MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I MOLECULES

Major histocompatibility complex (MHC) molecules are cell surface proteins expressed on almost all nucleated cells in the body. There are two types, major histocompatibility complex I (MHC-I) and major histocompatibility complex II (MHC-II), both encoded by a polymorphic set of genes located on chromosome 17 in the mouse. For MHC-I molecules, two subfamilies called classical (MHC-Ia) and non-classical MHC-I (MHC-Ib) molecules have been described. They are structurally similar, but differ in many features; for example, the former are highly polymorphic whereas the latter are more conserved. Generally, MHC-I refers to classical MHC-I unless mentioned otherwise. An MHC-I molecule consists of two

polypeptide chains, the α chain having three domains (α1, 2, 3) and a β_2-microglobulin chain (β_2m), which are linked non-covalently. Only the α chain is polymorphic and encoded by an MHC-I gene. Its α1 and α2 domains form a cleft capable of binding antigens, usually short (8-11 amino acids) peptides. The β_2m subunit is non polymorphic, encoded by the β_2m gene and associates with the α chain to help maintain the proper conformation of the MHC-I molecule, necessary for its expression on cell surface. β_2m knockout mice ($\beta_2m^{-/-}$), therefore, have greatly reduced MHC-I cell surface expression [53]. Studies in mice and human differ as mouse research is done on mostly inbred laboratory strains, allowing a reductionist approach in these studies.

In humans, MHC-I molecules are referred to as HLA (human leukocyte antigens) while classical MHC-I molecules in mice are termed H-2. The H-2 complex on chromosome 17 includes three MHC-I loci: D, K and L, each with multiple alleles. The H-2 haplotypes in various laboratory mouse strains have been characterized extensively and used in studying immunological processes, especially by mouse models obtained via breeding or transgenic technology. Homozygous inbred mice strains have unique H-2 haplotypes termed as e.g. H-$2^{a, b, d, k, q, s,}$ etc., where each haplotype refers to a particular combination of alleles at the K, D and L loci. The H-2 haplotype of the C57BL/6 mice (B6) is H-2^b, which contains K^b and D^b alleles, and a null allele at the L locus. Other examples of H-2 haplotype are H-2^d in Balb/c and DBA, which contain K^d, D^d and L^d alleles. Figure 2 shows the schematic structure of the H-$2D^d$ molecule and an engineered derivative, in which the α3 domain is derived from L^d. The latter transgene has been introduced in the DL6 mouse which is the focus of this thesis.

The MHC-I and MHC-II molecules play a crucial role in antigen presentation. When MHC-I molecules are produced inside the endoplasmic reticulum of the cell, they bind peptides synthesized within the cell, usually from endogenous proteins, but during infection, they can also load microbial (e.g. viral) peptides. These peptides are actively transported into the endoplasmic reticulum via transporters associated with antigen presentation or TAP molecules. Upon traffic to and expression on the cell surface, the MHC-I molecules present these peptides to $CD8^+$ T cells, whose T cell receptors can bind and dock to combine elements of the MHC-1 molecule and the peptide [54].

Another function of MHC-I molecules is to act as ligands for NK cell receptors such as Ly49 receptors (Ly49r). The different inhibitory Ly49 receptors bind MHC-I molecules, each with a certain allele specificity or at least allele preference, as described later [53]. This specific interaction forms the basis of the 'missing-self' hypothesis. These receptors will be discussed more in detail below, but it is pertinent to already here mention some structural aspects regarding their interaction with MHC-I. This binding usually requires a peptide in the antigen binding cleft, and although some peptides are more permissive for certain Ly49r, the latter do not distinguish peptides with the same fine specificity as T-cells. The Ly49r/MHC-I interaction is quite different from the T cell receptor/MHC-I interaction; in most cases the NK cell receptor docks to the MHC-I ligand close to the α1 α2 helices, but may also bind near the floor of the antigen binding groove close to α3 helix [53, 55, 56].

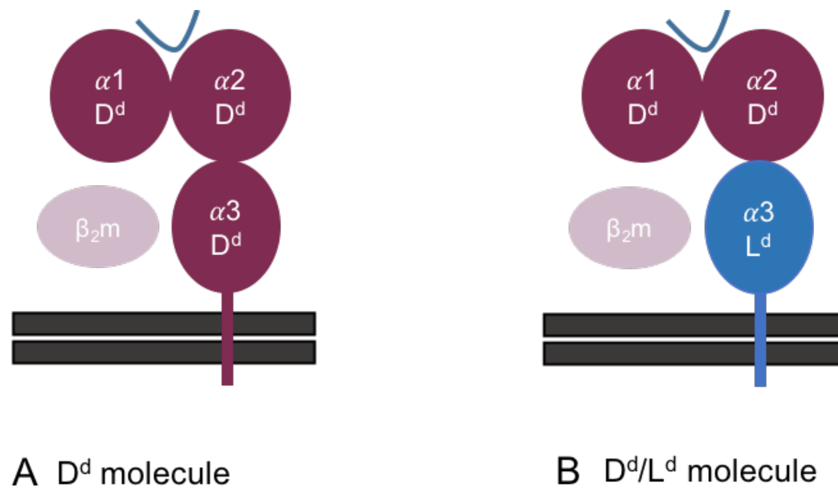


Figure 2. Schematic picture of MHC-I molecules encoded by different transgenes used in the thesis.

1.4 NATURAL KILLER CELL RECEPTORS

The NK cells receptors serve various functions including roles in development, maturation, education and effector function [57]. Many of these receptors are not limited to NK cells and may be expressed on other immune cells e.g. dendritic cells, T cells, etc. [58]. They include a variety of adhesion molecules as well as cytokine and chemokine receptors.

The main receptors involved in NK cell function are the inhibitory and activating receptors that take part in cognate interactions with other cells. NK cells possess a host of germ line encoded receptors, both inhibitory and activating in nature. These receptors comprise proteins from various families and with different specificities. Host MHC-I alleles influence the expression and function of some of these receptors, and in many cases, influence their cell surface expression levels as well (discussed further in the education section 1.6).

Many of the inhibitory receptors interact with MHC-I and similar molecules expressed by all healthy nucleated cells, while the activating receptors, in addition to MHC-I, interact with a host of endogenous or exogenous proteins related to stress, senescence, non-self, pathology, etc. Many ligands have been identified for both types of receptors, although they are not well characterized, especially for the activating receptors.

The NK cell recognition system for target cells depends on interaction between multiple NK cell receptors and the corresponding ligands on the target. The receptors for target recognition act in concert, adding up to a net signal resulting from the balance of activating and inhibitory receptors.

1.4.1 Inhibitory receptors

NK cell inhibitory receptors consist of various families. In mice, the major inhibitory receptor family belongs to the type II integral membrane C type lectin protein family. These NK cell receptors are known as Ly49 receptors (recently termed as klra family killer cell lectin-like

receptor, subfamily). They represent a multigenic family located on chromosome 6. In humans, they exist only as pseudogenes. Like the MHC-I complex, the *Ly49* gene locus is diversified in terms of alleles and ligand specificity, showing considerable differences among mouse strains, thus uniquely affecting immune responses in them [57].

Most of these receptors display some MHC-I allele preference, but the specificity is not limited to one MHC-I ligand. Moreover, the binding of one receptor to different MHC-I ligands can vary quantitatively e.g. the Ly49A receptor binds strongly to D^d. The Ly49C receptor binds strongly to K^b. The Ly49G2 receptor binds to D^d. The Ly49I receptor binds to K^b and D^b. There are also a number of weak interactions that have been described using different binding assays as well as functional assays e.g. Ly49C binds weakly to D^b, Ly49A to D^b, Ly49G2 to L^d, D^b, etc. [13, 59-65]. Another type of inhibitory receptors is NKG2A/CD94, which are dimers of natural killer type II transmembrane protein family with CD94. They recognize class 1b MHC-I in mouse i.e. non-classical Qa-1^b, which preferentially presents Qa-1 determinant modifier or Qdm, a peptide derived from the leader sequence of certain MHC-I alleles i.e. D^d and L [66].

The cytoplasmic domains of the inhibitory Ly49r contain immunoreceptor tyrosine based inhibitory motifs or ITIMs. Upon recognition of an MHC-I molecule by the receptor, these ITIMs are phosphorylated by Src kinases and recruit adaptor molecules like SHP-1 or SHIP-1 phosphatases, which can act by dephosphorylating substrates like Vav1, thus blocking the downstream activating pathways. Via inhibitory signal transduction, the lytic function, among others, is downregulated [29, 67].

In humans, the inhibitory NK cell receptors include proteins of the killer immunoglobulin-like receptor family (inhibitory KIRs e.g. KIR2DL, KIR3DL, recognizing specific classical 1a MHC-I/HLA alleles e.g. HLA-C), natural killer type II transmembrane proteins with an extracellular C-type lectin (CD159 family including NKG2A, NKG2C, NKG2E, etc. which form dimers with another C- type lectin CD94, recognizing non classical 1b MHC-I molecules e.g. HLA-E), etc. [29].

Other receptors with inhibitory function present on NK cells include KLRG1 (MAFA, binding cadherins) and 2B4 (CD244, binding CD48) in mouse and NKRP1 [68], [69] and other SLAM family members [70].

1.4.2 Expression pattern of inhibitory receptors

The Ly49 receptors are expressed in a variegated pattern, such that each receptor can be either on or off in a particular NK cell, and this correlates with transcription of the receptor gene. This is regulated independently for each of the *Ly49* genes and the consequence is that each NK cell may express none, 1, 2, 3, 4, 5 or even more different Ly49 inhibitory receptors. The expression pattern may be attributed to the presence of bidirectional promoters upstream of the Ly49 gene locus or to long-range methylation of the locus. Moreover, the probability for "on" differs e.g. in B6 mice 15% of NK cells are Ly49A^+, while 40% are Ly49C^+. The probability to express two Ly49r corresponds to the product of the probabilities for expression of each of these receptors (Ly49C and Ly49A: 0.15 x 0.40 = 0.06), referred to as “the product rule”. Expression is independently regulated for each of

the different Ly49 receptors, thus, leading to overlapping expression patterns. Similar rules apply to the expression of KIR in humans [13, 59, 71-74].

1.4.3 Activating receptors

In mice, NK cell activation receptors include some members of the Ly49 family, e.g. Ly49D (recognizing D^d allele of mouse MHC-I), Ly49H (recognizing m157 protein encoded by MCMV). NKG2D (CD134) is a member of the natural killer type II transmembrane family; it can bind to target cell ligands induced by stress or infections, such as Rae-1β, H-60, MULT1, etc. [75]. Other examples of activating receptors are NKR-P1C (NK1.1 or CD161.c, belonging to a family of receptors encoded in the mouse NK complex, NKC), NKp46, NKp30 and CD16 (low affinity Fc-γIII receptor). The latter allows NK cells to mediate antibody dependent cell mediated cytotoxicity (ADCC), a reaction that may be an important effector mechanism for many antibodies used as drugs to treat tumour patients. The ligands of the other mentioned receptors are less clear, although some candidates have been suggested (e.g. influenza virus hemagglutinin in the case of NKp46). It should be noted that normal, healthy cells are killed by NK cells if they lack MHC-I molecules, which means that certain ligands for activating NK cell receptors must be expressed constitutively. It is not clear whether such ligands represent low grade expression of the known ligands for activating receptors, or an additional set, of so far unknown group of molecules [29].

Many activating receptor families have short cytoplasmic tails, and signal via association with adaptor molecules like CD3ζ, FcεRIγ, DAP12, etc., which contain immunoreceptor tyrosine based activating motifs or ITAMs. The association with the adaptor molecule occurs via a charged residue in the cytoplasmic tail in the case of activating Ly49 receptors, interacting with DAP12. The adaptor molecules, in turn, recruit kinases like ZAP70 or syk, or other adaptor molecules like DAP10. Thus, the phospho-inositol tri phosphate PI3 pathway of cell activation is initiated [29, 30, 67].

The various co-activating receptors include adhesion molecules like CD2, CD62L, LFA-1, (CD11a, binding ICAMs), etc., and DNAM-1 (CD226) which may be both an activating receptor and an adhesion molecule. It binds to CD155 and CD112, and seems to be crucial for certain NK-tumour cell interactions, e.g. in melanoma [76].

In humans, NK cell activating receptors include some proteins of the killer immunoglobulin-like receptor family (activating KIRs e.g. KIR2DL, KIR3DL, recognizing specific classical 1a MHC-I/HLA alleles e.g. HLA-C), the NKR-P1A family, natural cytotoxicity receptors (NCRs e.g. NKp30, NKp44, etc.), CD16 (Fcγ receptors for the Fc end of IgG antibodies), natural killer type II transmembrane proteins (NKG2D or CD314 recognizing stress/altered molecules like MICA/B, Hsp90, Rae-1g, MULT1, H-60, ULBP, etc.), to name a few. For human NK cells, activation requires the involvment of at least two different activating receptors (exception is CD16) [77].

1.5 MISSING SELF AND OTHER RECOGNITION STRATEGIES

MHC-I alleles specifically interact with Ly49 inhibitory receptors on NK cells. This forms the basis of the missing self hypothesis [78]. During effector phase, NK cells interact with cells via both activating and inhibitory receptors. The final outcome of the interaction

depends on the balance between these signals. When interacting with a normal cell which has self MHC-I molecules and none or few activating ligands, the balance is tipped to the inhibitory side. On the contrary, when interacting with an altered/foreign cell having decreased or no self MHC-I molecules, the balance favours NK cell activation as explained by the 'missing self hypothesis'. The balance can also be altered by expression of proteins induced during stress, infections (especially viral), tumour transformation, senescence, etc. which bind to activating NK cell receptors [79]. This is referred to as 'induced-self recognition'. Current data suggests that some activating ligands seem to be constitutively expressed by normal cells. When an NK cell interacts with a normal cell which has self MHC-I molecules and none or few known activating ligands, the balance is tipped to the inhibitory side. An inhibitory signal would interrupt the activation signal via dephosphorylation of molecules critical for the activation pathway, and inhibits cytotoxicity. A change in the inhibitory signal (i.e. reduced MHC-I expression) may be sufficient to trigger effector function as one example of extreme situations. Another extreme is a target with normal MHC-I expression, but dramatically increased expression of activating cell surface ligands induced by stress; under these conditions, the balance is tipped to the activating side. One may imagine that the NK cell's decision may often be based on a combination of these changes at the target cell level, e.g. moderately increased expression of activating ligands, combined with moderately reduced expression of inhibitory ligands. Regardless of the details behind this altered balance leading to effector function, the latter may include cytotoxicity as well as cytokine secretion. During the maturation phase, the interaction between NK cell receptors, most importantly the inhibitory receptors, and MHC-I molecules set the activation thresholds and functional capacity of the NK cells, as discussed further below.

While infected and tumour transformed cells both may often increase activating ligands and reduce expression of MHC-I, there are certain situations, where a cell without any alterations in the activating ligands can be killed, simply because it lacks the critical inhibitory self MHC-I ligand e.g. allogeneic cells. When the absence or reduced expression of MHC-I play a decisive role, this is referred to as "missing self" recognition [80].

Missing self recognition is sensitive and enables NK cells to detect completely missing MHC-I molecules i.e. "general" missing self, e.g. in case of $\beta_2m^{-/-}$ cells, and partially missing MHC-I alleles i.e. "allele-specific" missing self, e.g. in case of allogeneic cells [81]. It explains both the recognition and killing of tumour cells as well as hybrid resistance, an occurrence where the F1 hybrid i.e. the offspring from a cross between two inbred strains, resists parental grafts, and which classical transplant immunology had failed to explain [13], as discussed below.

1.6 NATURAL KILLER CELL EDUCATION

Classical laws of transplantation are based on the notion that an F1 hybrid contains all the histocompatibility antigens present in the parental mice. The latter would reject grafts from the F1 but not vice versa. These laws were based on and predicted T cell responses [82, 83]. However, T cell independent rejection of parental bone marrow grafts was seen in F1 hybrids in case of mismatched MHC-I molecules, and NK cells were eventually shown to

be involved in this. Several models were presented to explain this peculiar type of rejection, e.g. recessive Hh antigens [84-86]. Later, studies in transgenic D8 mice having a D^d transgene on a B6 (K^b+D^b) background, showed that non-transgenic B6 (K^b+D^b) bone marrow or lymphoma [87] was rejected in such mice contrary to classical transplantation laws. This was a significant observation as it demonstrated that the rejection was actually due to influence of host MHC-I genes [78, 88].

These studies suggested that MHC-I molecules affected the NK cell specificity development. This became clear when the gene targeting technology could provide MHC-I deficient mice. Since self MHC-I molecules are important in protecting the target from NK cells, it could be assumed that in an MHC-I deficient mouse, all cells would be more prone to lysis by their own NK cells. However, in β_2m deficient mice, this was not the case. In such a setting, the animal's own NK cells did not kill any MHC-I deficient cells *in vivo* or *in vitro* [89, 90]. This led to the hypothesis that the presence (or absence) of self MHC-I in the host specifically determines the outcome of individual effector NK-target cell interactions. This effect has been termed as education [91].

For most immunologists, the term education equals the term deletion i.e. deletion of clones in a selection process. However, other possibilities could not be excluded.
Previous observations in MHC-I mosaic mice (which have a mixture of cells with respect to MHC-I in that some cells express D^d molecule and some do not) showed that NK cells, which maintain tolerance to cells with a certain MHC-I phenotype *in vivo*, lose that tolerance upon a change in environment and can lyse those cells. This change in NK cell specificity showed their adaptability to changed MHC-I expression in the environment [92]. Thus, it was concluded that although NK cells become tolerant to all MHC phenotypes present in the host, tolerance to one of them can be broken when cells with this phenotype are removed from the environment. This suggested that NK cell tolerance is reversible and may be due to mechanisms leading to a temporary anergy-like state in the cells, and not from clonal deletion that would have removed the cells altogether.

This was formally proven in 2005, independently by the groups of Yokoyama and Raulet. It was known that-inhibitory receptor expression is variegated. Some NK cells have self specific inhibitory receptors in the host, while some do not. The two groups showed that if the NK cells do not express any self-MHC-I specific inhibitory receptors, they do not become responsive; their activation threshold is maintained high and they become "hyporesponsive", without having to be inhibited by recognition of MHC-I ligands. But if they do express self-MHC-I specific inhibitory receptors, they become responsive depending on the cognate self-MHC-I [93]. The hyporesponsive NK cells maintain self tolerance, which is MHC-I independent. They do not respond well in assays based on activation of NK cell receptors or towards MHC-I deficient healthy cells [93, 94]. Thus, NK cells that do not meet a cognate MHC-I ligand are not deleted but kept in a hyporesponsive or unlicensed state. This was also observed in human NK cells [95].

Thus, during the maturation phase, the interaction between NK cell receptors, most importantly the inhibitory receptors, and MHC-I molecules set the activation thresholds and functional capacity at the single NK cell level.

Phenotypically, no marker for education has been identified as yet, but functionally, it is measurable in a number of ways, at several levels [67, 96]. In mice, at the NK system level, educated NK cells can mount a strong rejection response to an *in vivo* challenge with MHC-I^- cells (measurable by an *in vivo* assay for NK cell mediated rejection which is described later). This can also be tested using *in vitro* killing assays. At the cellular level, the functional capacity of an NK cell can be analysed using single cell responsiveness assays. Educated NK cells degranulate and produce more cytokines e.g. IFN-γ, as compared to uneducated cells when exposed to antibodies against activating receptors [67, 96].

In addition, the host MHC influences the frequency distribution of subsets with different patterns of Ly49r expression. This influence is exerted at the system level.

In MHC-I deficient mice, no NK cells have seen MHC-I molecules and many of them express multiple inhibitory receptors, whereas in mice with MHC-I expression, due to interaction with specific MHC-I alleles, the NK cell receptor repertoire is 'skewed' towards more single receptor positive NK cells, particularly those specific for self MHC-I [97, 98].

Another observation is that cell surface expression of an inhibitory receptor on NK cells is reduced when its ligands are expressed in the host [99, 100]. This can be caused by cis and trans interactions with MHC-I, but cannot be used as a marker of responsiveness [29, 101, 102].

1.6.1 Models for education

Based on the influence of MHC-I on NK cell function, three models of NK cell education have been proposed:

1) The Licensing model suggests that NK cells are inactive by default and are allowed to mature to full functional potential depending on interactions with self MHC-I. Evidence shows that signalling by ITIM containing receptors is required for licensing to take place. Unlicensed NK cells remain self tolerant, even if they lack inhibitory MHC-I receptor [68, 93].

2) The Disarming model states that mature NK cells are active by default, but if they fail to interact with self MHC-I due to lack of specific receptors or ligands, they become selectively disarmed. This NK cell anergy would be a mechanism to maintain self-tolerance [69, 94].

3) The Rheostat model hypothesizes that NK cells can be continuously and reversibly regulated so that they become more or less responsive, depending upon the strength (and frequency) of encounters with cognate MHC-I molecules [96]. It reconciles both the licensing and disarming models, and is described in more detail below.

1.6.2 The rheostat model

NK cells sense inhibitory signals through their Ly49/NKG2 receptors, which have different allelic specificities and affinities for MHC-I. Thus, MHC-I alleles differ in their efficiency in

educating NK cells and the number and type of host MHC-I alleles quantitatively influence NK cells expressing individual Ly49 receptors [59, 103, 104]. The "sum" of inhibitory signals received by an individual NK cell during education, tunes the downstream activation pathways quantitatively, synonymous to a "rheostat" controlling an electrical circuit [96] (Figure 3). Tuning up results in low activation threshold and high responsiveness, thus normal cells would only be protected by adequate MHC-I expression. On the other hand, tuning down results in an increased activation threshold towards normal cells.

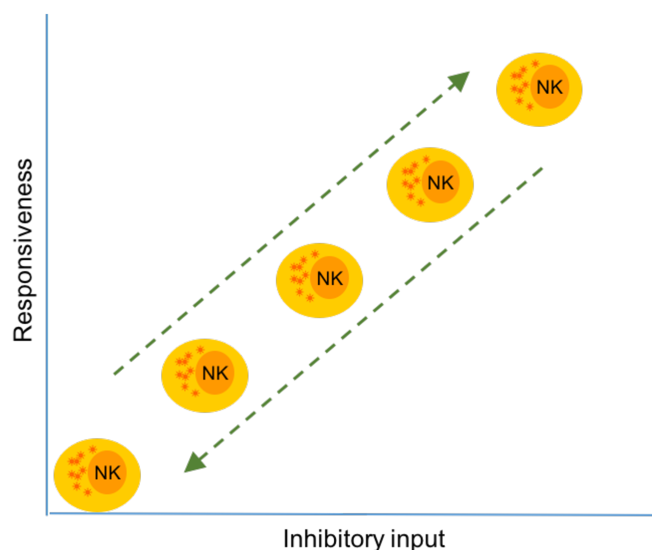


Figure 3. The rheostat model. NK cell responsiveness is tuned at a single cell level.

This process determines the frequency of responsive NK cells at the population level and also control the strength with which, each responsive NK cell exerts its effector functions. Thus, for quantitative effector responses like degranulation and cytokine production, the NK cell increases its responsiveness, resulting in more IFN-γ production per cell [96]. These two effects together lead to a more efficient *in vivo* missing self response at the organism level. This can be interpreted as the 'educating impact' of a given combination of MHC-I [59, 96].

There are several lines of data suggesting that education is quantitative and reversible rather than a digital on/off feature. Reversible tuning is consistent with observations in MHC-I mosaic mice as described above, as well as with other *in vitro* and *in vivo* studies [105] [106]. The 'Licensing' and 'Disarming' models can be reconciled with each other in the rheostat model albeit with modifications, where the rheostat model reflects bidirectional tuning, up or down, of NK responsiveness as opposed to switching the cell on or off. Even after the maturation in bone marrow, NK cells would continuously undergo small licensing or disarming steps. Thus, mature NK cells constantly adjust to the MHC-I environment by continuously tuning their responsiveness upwards or downwards, depending on the net inhibitory input of the environment, to maintain optimal responsiveness with respect to 'missing self' recognition while preserving self tolerance [67, 96].

Licensed NK cells or NK with a majority of Ly49 (inhibitory) receptors specific for self MHC-1, respond to minor changes in MHC-1 repertoire/expression e.g. in tumours/transplants. Unlicensed NK cells are not non-functional cells. Although they are not responsive to normal cells in the host environment, they have been shown to respond vigorously to infections. They are dormant but can be awoken by increased activating signals e.g. in case of infections like MCMV or *Listeria* [94, 107].

The rheostat model states that this is a reversible and quantitative phenomenon, depending on the amount of signals a cell receives. Assuming NK cells incorporate this input over several cell encounters, it may also depend on the ratio between cells with and without a critical MHC-I ligand. This can occur under conditions of chimaerism after haematopoietic transplantation. One major aim of this thesis was to address whether education for NK cell responsiveness and missing self rejection *in vivo* is influenced quantitatively by the frequency of cells with a given MHC-I ligand.

1.7 NK CELLS IN NATURAL RESISTANCE AGAINST CANCER

Evidence that NK cells can contribute to natural resistance against transplantable tumours was presented already soon after the discovery of NK cells in the 1970s. Kiessling et al. showed that in different F_1 hybrid strains of A/Sn mice, the natural resistance against the semi syngeneic YAC lymphoma cells correlated with the NK cell activity against the same tumour cells *in vitro* [108]. They also demonstrated such resistance in athymic nude mice and mice treated with thymectomy, irradiation and reconstitution of bone marrow cells, arguing against a role for T cells [109]. These correlative studies were eventually followed by more direct studies based on NK cell depletion with antibodies such as anti-asialo-GM1 and anti-NK1.1, both of which could abrogate natural resistance against transplantable tumours [110-113]. Experiments along this line also demonstrated that NK cells were particularly efficient in protection against haematogenous metastasis, e.g. in studies of intravenously inoculated B16 melanoma cells [114].

The early studies where based on long term evaluation of growth (or metastasis) of tumours. These experiments can cause suffering of animals, even if ethical guidelines on maximal tumour size is followed. Furthermore, they are time consuming and the outcome can be influenced by a variety of factors. Several different assays for direct monitoring of NK cell mediated killing *in vivo* of intravenously inoculated (radio labelled or fluorochrome labelled) tumour cells have therefore been developed, including some new protocols presented in the results part in the present thesis [115-117]. Such methods have been particularly useful to test the influence of MHC-I genes in missing self based recognition and resistance against tumours.

More recently, it has been addressed whether NK cells contribute to natural resistance against tumours in their host of origin (autochtonous host). These studies have used chemical carcinogenesis or transgenic models based on oncogene driven tumour development. [51, 118], and show an important role of NK cells in host protection. NK cells in immunosurveillance against tumours in humans is of course much more difficult to study, although attempts have been made in correlative studies. A role of NK cells was suggested from a study in which NK cell levels were studied longitudinally with a 11 year interval using a functional cytotoxicity readout, which showed that a high level of cytotoxic activity among

peripheral blood lymphocytes was associated with a lower risk of cancer [119]. Similarly, high numbers of tumour infiltrating NK cells correlate to higher survival in several solid tumours [120, 121].

1.8 NK CELLS IN IMMUNOTHERAPY AGAINST CANCER

Regardless of the role of NK cells in natural resistance against cancer, they are interesting in the perspective of immunotherapy against cancer. This has been exploited in experimental studies in mice and NK cells are now also being studied extensively for the purpose of clinical use. Immunotherapy is a rather new arm of cancer treatment along with conventional methods like surgery, chemotherapy, radiation, etc. NK cell activity can be harnessed or modified in various ways to induce or improve their therapeutic efficacy in cancers including, including cytokine activation, transfer of cells, antibody mediated effects, genetic engineering or combination of these different methods.

1.8.1 Cytokine activation of NK cells

NK cells can be activated by several cytokines, such as IFN-β, IL-2 and IL-15. The latter can also be used to expand and maintain NK populations, *in vivo* and *in vitro*. The first direct studies of NK cells in immunotherapy were done by S. Rosenberg and colleagues using IL-2 activated mouse spleen cells or human PBMC [122, 123]. These cells were referred to as lymphokine activated (LAK) cells and proposed to represent a novel immune cell population, but later studies showed that the majority of the effector cells in these cultures were activated NK cells [124]. After activation and expansion *in vitro*, the cells were transferred back to the NK cell donors (tumour bearing mice or cancer patients), with or without concurrent treatment with IL-2 injections. This approach resulted in some tumour regression effects also in clinical situations, however, there were severe side effects (e.g. pulmonary oedema). IL-2 treatment alone is still being used in treatment of certain cancers [125, 126]. IL-2 can also be combined with drugs, for example in case of AML (acute myeloid leukaemia), the combined use of low dose IL-2 with HDC (histamine dihydrochloride), has been shown to reduce the risk of relapse significantly, post remission [127, 128].

These pioneering studies have then been further developed, and today a number of protocols are being used to generate large numbers of NK cells *ex vivo* [129]. GMP (good manufacturing practice regulations) compliant protocols for clinical grade NK cells are being developed and tested for efficacy and safety, using bioreactors with cytokines and feeder cells. CIK (cytokine induced killer cells) generated by use of IL-15 and IL-21 for NK cell differentiation and maturation do not need priming *in vivo*, by exogenous IL-2 administration [130, 131].

A recent approach in this field is to exploit the features of so called memory NK cells, e.g. cells which after activation or prolonged stimulation (with cytokines or CMV), show increased efficacy and survival. Such memory-like NK cells have increased functional capacity for long-term (weeks) on re-encountering cytokines or tumours (leukaemia targets), be it licensed or unlicensed NK cells [132].

1.8.2 Harnessing allo- and missing self recognition in haematopoietic transplantation and adoptive transfer of NK cells

An effective post-remission therapy for acute leukaemia patients with high risk of relapse, is allogeneic haematopoietic stem cell transplantation (HSCT)/HLA-haploidentical HSCT. Extensive matching of HLA is required to prevent rejection and graft versus host disease i.e. GVHD. In cases when matched donors were not available, attempts were made to use partly matched donors, for example haploidentical ones, where only one HLA haplotype was matched. This is often the case if the donor is a parent of the recipient. Earlier, it was observed that haploidentical (HLA haplotype mismatched) transplantation elicited allo-responses, both rejection and GVHD. The GvL effect of T cells was overridden by their GVHD effect. To counteract this, high dose, extensively T cell-depleted peripheral blood haematopoietic progenitors were transplanted, with no post transplant immunosuppression. This brought focus on the alloreactive role of NK cells, in eradicating AML [133].

The application of NK cell mediated missing self reactivity in immunotherapy was pioneered by Velardi et al. in studies of haplo-identical HSCT i.e. transplantation between two persons sharing one HLA haplotype (often parent to child transplants). Ruggeri et al. observed that in such HLA mismatched HSCT cases, where there was a predicted donor versus recipient NK allorecognition (i.e. allele specific missing self recognition), donor NK clones obtained from transplanted recipients killed recipient target cells *in vitro*, but did not cause GVHD (graft versus host disease). These KIR ligand mismatched donor stem cells also showed better engraftment [134]. In a follow up study, it was shown that NK cell alloreactivity in haploidentical HSCT could not only protect from graft rejection and GVHD, it could also prevent leukemia relapse in AML patients. Furthermore, in a mouse model, infusion of KIR ligand mismatched NK cells before transplant, not only correlated with reduced leukaemia relapse and fewer cases of graft rejection and GVHD, but also lowered the risks associated with conventional conditioning regimens followed in transplant protocols [135]. The results of the HSCT were confirmed by several studies [136-139]. However, other studies with mainly unrelated partly matched BM donors did not reveal an advantage of donors with potential for NK cell missing self reactivity in the graft vs. host direction [140-143]. The reason for these discrepant results (reviewed by Witt and Christiansen [144]) may be due to differences in protocols. It appears that the beneficial effect of NK cell missing self reactivity requires exhaustive T cell depletion and a high number of haematopoietic stem cells in the graft. The *KIR* genes play an important role in this, suggesting that KIR allele typing may be important in selecting a donor [145]. It has been seen that the responding patients have more alloreactive donor NK clones [146].

Alloreactive NK cells (i.e. those that express KIRs, which do not recognize HLA class I molecules on recipient cells) from haploidentical donor HSCT, prevent graft rejection in the absence of GVHD, and reduce relapse by killing patient dendritic cells, T cells and leukaemia blasts/residual leukaemia cells (especially in AML patients) [147]. A correlation has been seen between the size of this allreactive NK cell population and clinical outcome [148]. Thus, allogeneic NK cells have anti-tumour potential like T cells, do not cause GVHD and do not require HLA matching of donor and recipient [145], [129]. To further improve this, new approaches are being studied e.g. to expand them *in vitro* [149] as well as standardizing the

protocols for clinical generation of alloreactive NK cells [150] for improved clinical outcome [151, 152].

Early results from NK cell infusion based clinical trials have shown feasibility and safety without the occurrence of cytotoxicity or GVHD [131, 153, 154], with remission and disease free survival seen in some patients with haematological malignancies. This safety was also seen in case of haploidentical NK cell infusion, after relapse in AML or MDS (myelodysplastic syndrome) patients post allogeneic HCT (haematopoietic cell transplantation) [145]. Similar results have been obtained from studies for adoptive NK cell based therapy from haploidentical donors [155]. In AML patients after HLA mismatched allogeneic HSCT, post transplant adoptive transfer of donor NK cells, especially activated NK cells, showed better results than un manipulated donor lymphocyte infusions (DLIs) [156]. The efficacy of this method of treatment is further enhanced by the milder protocols involved, such as a lack of need for irradiation. In elderly AML patients, who are not eligible for HSCT, adoptively transferred haploidentical NK cells, especially in high numbers, (purified alloreactive NK cell infusions) post complete remission, has shown to be effective and safe [146, 157]. In paediatric patients of AML, there was a higher relapse and lower survival compared to other paediatric cancers. NK cell adoptive transfer reduces the need for chemotherapy and eradicates AML blasts [158]. The objective is to maximize NK cell alloreactivity for achieving effective GvL and no GVHD [145, 159, 160].

Studies have investigated the effects of NK cells in inducing graft versus leukaemia (GvL) and GVHD not only in case of AML, but also other haematological diseases including sarcomas, carcinomas, brain tumours, etc. [131]. The results from solid tumour patients, however, have not been so positive [155].

1.8.2.1 Cancer treatment based on antibodies

1.8.2.1.1. Possible contribution of NK cells via ADCC in different situations

As noted above, NK cells can use the CD16 receptor to mediate ADCC against antibody coated target cells. There are a number of antibody based drugs used in oncology today, most of which have target molecules expressed on tumour cells. Surprisingly little is known about the exact mechanisms behind therapeutic efficiency, but this is not easy to study in the human. Using mice with different gene targeting induced deficiencies in Fc receptor (FcR) expression, it has been clearly demonstrated that ADCC play a major role in certain anti-tumour effects induced by administration of antibodies [161]. It has been proposed that some of the therapeutic effects seen in clinical treatment may also depend on ADCC and that NK cells therefore play a role. One example is the drug Herceptin (antibodies against the HER-2/Neu growth factor receptor [162] used to treat some forms of breast cancer. There are studies suggesting ADCC as mechanism here, because patients with a polymorphism for the CD16 receptor respond better and thus, these polymorphisms may carry a predictive value for progress [163, 164]. It is, of course important to understand if NK cell mediated ADCC is involved in certain therapies, because the efficacy of the treatment can then be improved by modifying the antibody (e.g. higher affinity to Fc-receptor) or by combining it with other NK cell activating modalities, be it cytokines or other antibodies.

A more recent development in this area is the use of genetically engineered antibodies, generating bi- or tri-specific antibody complexes which target several different molecules on the tumour cells and the effector cells [165]. In this approach, the antibody complex is usually designed to target one or more of the activating receptors on NK cells.

1.8.2.1.2 Blockade of inhibitory receptors

1.8.2.1.2.1 KIR/Ly49r blockade

Another way to harness "missing self recognition" for immunotherapy is to block inhibitory receptors. According to the hypothesis, this should induce NK cells to kill tumour cells but possibly also normal cells expressing MHC-I molecules, since one normal guard to ensure self tolerance is blocked. The rationale to explore this approach for immunotherapy was that the threshold for NK cell reactivity to tumour cells might be easier to overcome, due to different properties of the tumour cells (e.g. the expression pattern of activating ligands). There was thus a possibility for "therapeutic interval" where inhibitory receptor blockade would result in anti-tumour activity, without attack of normal cells.

Koh et al. were the first to demonstrate that inhibitory Ly49 receptor blockade in mice could result in augmented tumour resistance [166]. These authors demonstrated that anti-tumour activity of NK cells increase *in vivo* as well as *in vitro*, after inhibitory KIR receptor blockade. This has been confirmed by follow up studies showing that short term effect of this approach can be utilized to eliminate tumour cells before autologous transplant, without long term effects [167-169]. An anti-KIR human antibody was generated and in humanized mice, it mediated enhance NK cell lytic activity against AML tumour cells as well as stable blockade and long term survival [170]. Using an anti-KIR antibody in combination with anti-CD20 antibody (rituximab), increased the anti-lymphoma killing capacity of the NK cells *in vivo* and *in vitro* [171].

Vahlne et al. in our laboratory were the first to explore whether the treatment really induced NK cell mediated killing of tumour cells *in vivo*, and also whether NK cell attack on normal cells occurs in parallel [168]. This study used an *in vivo* assay to measure NK cell killing of fluorochrome labelled tumour cells and normal cells, in B6 mice that had been treated with $F(ab')^2$ fragments of an antibody against Ly49C and Ly49I receptors (5E6), i.e. a similar protocol to the one originally used by Koh et al. [166]. The inhibitory receptor blockade could indeed induce augmented *in vivo* killing of MHC-I expressing RMA lymphoma cells, while there were no signs of attack on MHC-I expressing B6 spleen cells, even after prolonged treatment (up to 13 weeks). Nor was there any killing of normal cells observed if mice were treated with the NK cell activating cytokine IL-2 in parallel with the inhibitory receptor blockade, even if this treatment further augmented the induction of lymphoma cell killing. Extensive investigations on haematological parameters and histopathology of a variety of organs revealed no signs of autoreactive attacks in mice treated up to 13 weeks. This study thus supported that there is a therapeutic interval for inhibitory NK receptor blockade. However, the rather modest augmentation of tumour cell killing, and the strikingly robust tolerance to normal cells observed in treated mice raised the question whether the perturbed sensing of MHC-I by inhibitory receptors might have more complex consequences than blockade of the inhibitory self recognition during the effector target interaction. This is

further explored in the results and discussion, as it is one of the questions posed in the studies of this thesis.

A full understanding of all consequences of perturbed sensing of NK cell inhibitory receptor blockade is essential in order to understand and develop efforts to apply this concept clinically. Several clinical trials have been completed or initiated, based on these initial observations. A number of studies and some clinical trials are under way to study the efficacy of different KIR blocking antibodies alone or in combination, and their outcomes e.g. in multiple myeloma, lung cancer, etc. [172-174].

1.8.2.1.2.2. Blockade of inhibitory receptors other than KIR/Ly49 Receptors

It has been observed that a pan-HLA blocking antibody makes AML and ALL lymphoblasts susceptible to killing by KIR mismatched NK cells. Additionally, blocking inhibitory receptors increases killing as does double blocking of other inhibitory receptors like LIR-1 and NKG2A. Combined with KIR blockade, blocking of LIR-1 and NKG2A shows potential for NK cell therapy in acute leukaemias (like 5E6 i.e. anti-Ly49C/I F(ab')^2) [175].

T cell intervention has been the more common form of immunotherapy e.g. T cell checkpoint blockade [176-179]. Tumour or associated cells may suppress immune cells *in vivo*, via inhibitory soluble factors, cytokines or by engaging immune check-point molecules (e.g. PD1/PDL-1) [148, 180, 181].

The benchmark of immunotherapy i.e. CD8 T cell checkpoint inhibitors (CTLA-4, PD-1/PD-L1) cause expansion and activation of tumour-specific T cells to circumvent tumour induced immunosuppression and eliminate transformed cells. This leads to dramatic anti-tumour responses in some patients with advanced melanoma or lung cancer. Several other forms of cancer are now evaluated with these as well as novel agents for checkpoint blockades. This is a very promising area, in particular when the new drugs are combined with each other or with established therapies. Possible limitations are autoimmune side effects e.g. colitis. More research is required to predict anti-tumour responses and prevent side effects [157, 177, 178].

1.8.2.2 Genetically engineered NK cells (including CAR)

Modified NK cells can be genetically engineered transiently via mRNA electroporation, or permanently by viral transduction. The gene modifications include introduction of activating receptors, silencing of inhibitory receptors or redirecting NK cells to tumour killing via CARs (chimaeric specific antigen receptors) to enhance their specific cytotoxicity or to improve survival by autocrine cytokine stimulation *in vivo*. The CAR therapy has originally been developed for T cells, and appears very efficient in treatment of B cell lymphomas [182]. In theory, NK cells should be possible to be used also; they may even represent some advantages, and it has been suggested that NK cells are "..the better CAR-drivers" [183]. Cytokine production by NK cells can be induced to promote *in vivo* expansion [129], these genetic modifications enhance NK cell function and target specificity [131, 184, 185].

1.9 BONE MARROW CHIMAERISM IN THE CLINIC AND IN EXPERIMENTAL MODELS

1.9.1 Clinical studies

Chimaerism refers to a situation where an organism is composed of two or more different populations of genetically distinct cells, derived from different zygotes. Chimaerism can be inborn or induced, e.g. by transfusion or an allogeneic haematopoietic stem cell transplant. The latter procedure is a life saving procedure that is used for example in haematologic malignancies, congenital immune deficiencies and metabolic disorders. It is estimated that more than 50,000 transplantations are performed every year worldwide. For successful engraftment, harsh preconditioning usually involving side effects, is used. In case of radiation susceptible malignancies (e.g. haematological like CML (chronic myeloid leukaemia) and AML), whole body lethal irradiation and/or cytostatic drugs are used. Despite these measures, diseased tissue may remain and lead to relapse of malignancy (MRD; minimal residual disease). Another complication is GVHD, which is frequently seen in mismatched bone marrow transplantation [186]. A number of studies have described chimaerism associated with clinical transplantation and also how this condition affects tolerance in the T cell compartment. [187, 188].

Stable donor chimaerism (sometimes defined as >95% of donor derived haematopoietic cells), is aimed for in allogeneic bone marrow transplantation. High levels of residual recipient T cells may be related to graft rejection [189] and malignancy relapse. Conversely, correlations between high donor T cell chimaerism and GVHD have been reported [190]. However rarely, stable mixed donor/host haematopoietic chimaerism (hereafter referred to as mixed chimaerism) is established (5-95% host derived haematopoietic cells) [191, 192]. In the case of stable mixed chimaerism in patients with non-malignant diseases, mixed chimaerism did not correlate with any adverse effects, suggesting that tolerance had been established [191, 192]. In addition, mixed donor chimaerism has been reported in patients receiving double umbilical cord blood transplantation [193], as well as in patients who have undergone repeated bone marrow transplantations [194].

In organ transplantation, for example kidney transplants, methods to induce transplantation tolerance have been widely studied. If successful, the need for lifelong immunosuppression or harsh conditioning can be bypassed. One approach is to achieve mixed haematopoietic chimaerism i.e. engraftment of donor haematopoietic stem cells (especially HLA-mismatched) into minimally preconditioned recipients, leading to transplantation tolerance [187, 195, 196]. Studies of tolerance induction in mixed chimaeric mice (and other animals) have been done, along with early clinical trials [187].

Results from recent clinical trials show promise where BMT (bone marrow transplantation) from living donors was given to kidney transplant recipients leading to mixed chimaerism and allograft tolerance in MHC matched and mismatched transplants. This reduced the need for immunosuppression [187, 195]. Mixed chimaerism has been reported to induce donor specific tolerance, where in the absence of immunosuppression and extensive conditioning, there is less risk of rejection. Even if chimaerism is transient (primate and human studies), the benefits achieved are similar to those from durable chimaerism (murine studies) [196].

It is thus clear that mixed chimaerism can affect T cell tolerance. In order to use this optimally, the complex relationship between chimaerism and tolerance i.e. the central and peripheral mechanisms, both deletional and regulatory, which lead to tolerization in mixed chimaeras, need to be explored and understood in order to improve efficacy, introduce modifications and remove limitations faced in clinical transplant immunology. Not only that, it gives us a chance for better understanding of tolerance through mixed chimaerism [196]. Long term NK cell tolerance after allogeneic HSCT that was either KIR ligand matched or mismatched, was assessed in a study. It was found that the donor ligands determine NK cell education after transplantation, leading to an education pattern similar to that of the donor, which was stable for over 3 years post transplant ([197].

Mixed haematopoietic chimaerism can occur in humans without intentional transplantation or transfer of cells as a part of a medical procedure, but rather initiated during pregnancy [198]. Natural chimaerism is observed in several species of protists, plants, invertebrates, vertebrates and mammals. It may occur even after "natural transplantation" of somatic cells between adult individuals [199].

Natural haematopoietic mosaicism has also been described in humans, although to my knowledge, not with respect to MHC expression. Genetic mosaicism refers to the presence of two (or more) populations of cells with different genotypes, although they originated from a single fertilized egg. One form observed in humans is cytogenetic mosaicism, where a proportion of all haematopoietic cells carry certain large chromosomal anomalies that have arisen due to somatic mutations or genomic instability. The frequency in the population of such clonal mosaicism was in one study reported to be low (<0.5%) up until 50 years of age, but 2–3% in the elderly [200].

In the strict definition, genetic mosaicism should be based on cells with different genetic or chromosomal constitution, but the term is frequently used in the context of X-chromosome inactivation in females, who in each tissue carry a mix of cells where either the paternal or maternal X-chromosome is inactivated. This can be regarded as epigenetic mosaicism leading to genetically identical cells presenting a mosaic expression pattern. The main mouse model used in this thesis, DL6 mice, may represent an (non X-linked) epigenetic mosaic with respect to expression of an MHC class I transgene.

1.9.2 Experimental studies

As noted above, allogeneic bone marrow transplant is a life saving procedure in the clinic. Furthermore, irradiation bone marrow chimaeras have been, and still is an important experimental tool to study tolerance and education of immune cells. There is a vast literature where bone marrow chimaeras have been used to study T cell development, including positive and negative selection as well as peripheral mechanisms of tolerance [201-206]. The discussion here will focus on the use of bone marrow chimaeras to study regulation of NK cell development and tolerance. Already in 1977, Otto Haller et al. [207] showed that genetically determined levels of NK cell activity was a property intrinsic to the bone marrow, and not of the host, by doing crisscross bone marrow transplantation between MHC-I syngeneic mouse strains with high and low NK cell activity. Later, the generation of $\beta_2m^{-/-}$ to B6 bone marrow chimaeras showed that NK cell missing self reactivity was determined by

the $\beta_2m^{-/-}$ bone marrow [89]. In mixed allogeneic chimaeras, NK cells have been seen to be specifically tolerant to the donor and the host [208].

A role of the non-haematopoietic compartment for NK cells has also been shown. Transplantation of B6 x BALB/c F1 bone marrow into B6 hosts followed by assessment of rejection of lymphoid cells suggested that host cells rendered NK cells less responsive to B6 cells [209]. Furthermore, bone marrow chimaeras where pure B6 or $\beta_2m^{-/-}$ bone marrow or a mix of B6 and $\beta_2m^{-/-}$ bone marrow was transplanted into irradiated B6 or $\beta_2m^{-/-}$ hosts showed that presence of $\beta_2m^{-/-}$ cells in the host or in the transplanted bone marrow rendered NK cells tolerant to the $\beta_2m^{-/-}$ phenotype [105, 210]. In another study, mixed chimaeras were generated in B6 mice using B6 and $\beta_2m^{-/-}$ bone marrow. The tolerance against $\beta_2m^{-/-}$ cells was lost by NK cells, over time and the $\beta_2m^{-/-}$ cells were rejected. The tolerance was broken even faster in the presence of MCMV infection, showing that inflammation can play a role in breaking self tolerance [39]. Recently, during the final part of the work in this thesis, Tovbis Shifrin et al. reported that haematopoietic and non-haematopoietic cells regulate NK cell tolerance differently. During infections, tolerance imparted by the non-haematopoietic cells is stronger than that induced by the haematopoietic cells [211].

In an attempt to assess and quantify the requirement for cells with a partial missing self phenotype in mixed bone marrow chimaeras, mixes of B6 (D^{d-}) and D8 (D^{d+}) bone marrow were transplanted to irradiated D8 hosts. The results suggested that presence of low numbers (<20%) of D^{d-} cells, reduced but failed to completely abrogate outgrowth of D^{d-} lymphoma cells [212]. This question was further investigated in the present thesis work.

It should finally be noted that NK cell education and tolerance can be studied also in mice with mosaic MHC-I expression, e.g. where the haematopoietic (and non-haematopoietic) compartments are composed of cells with different MHC-I expression, despite being genetically identical and derived from the same zygotes. The mosaic DL6 model has already been briefly introduced in the section on NK cell education above. It will be described extensively in the results and discussion section, as it forms the basis of the major theme in this thesis.

2 AIMS

General aim:

To understand the influence of a perturbed sensing of MHC-I molecules in the host environment, on NK cell education for missing-self responses. In particular, to understand the effect of a mixed MHC-I expression in the environment as a model for optimizing NK cell based immunotherapy strategies in treatment of tumours.

In order to approach this general objective, we have used transgenic and chimaeric mice as models for clinical situations where a mixed MHC-I host environment may occur, such as in bone marrow transplantation or adoptive transfer. More specifically, we have used transgenic mouse strains on H-2b (K^bD^b) background where a D^d transgene is expressed in all cells (D8 and DL1 mice) or in a sub fraction of the cells (DL6 mice), as well as bone marrow chimaeras based on these strains. We were particularly interested in quantitative aspects of education, tolerance and responsiveness. Furthermore, we were interested in reactivity to three types of cells: those expressing a complete set of MHC-I genes (vis-à-vis the host) as well as cells lacking one MHC-I allele (the D^d transgene: "allele specific missing self" reactivity) and cells with no MHC-I expression at all ("general missing self responsiveness"). We have also used mice where perturbed MHC-I sensing of the environment is induced by antibody mediated blockade of inhibitory receptors on NK cells.

Specific aims:

The following specific aims emerged during the development of the work:

- To analyse the kinetics of the effects of perturbed MHC-I sensing via NK cell inhibitory receptor blockade on missing-self reactivity.
- To further characterize and develop a previously described transgenic mouse model (DL6) encompassing mosaic expression of an MHC-I gene (D^d) [92], and in particular, to identify mice with high frequencies (>85%) of D^d expressing cells.
- To develop refined *in vivo* assays for NK cell mediated rejection, to allow monitoring of three target cells simultaneously.
- To determine a quantitative threshold for NK cell tolerance to "allele specific missing self", in terms of the minimal frequency of ligand deficient cells required to induce tolerance in hosts with mosaic MHC-I expression.
- To determine whether NK cell responsiveness against "general" missing self is influenced quantitatively by the proportion of cells with and without a given MHC-I ligand in mice with mosaic MHC-I expression.
- To determine whether mosaic MHC-I expression exclusively in the non-haematopoietic host environment is sufficient to influence NK cell tolerance to "allele specific" missing self and "general" missing self responsiveness.

- To establish a model for generation of mixed (D^{d-} vs. D^{d+}) bone marrow chimaeras with high frequencies (>85%) of D^{d+} cells, and with NK cell function maintained.

- To determine whether mosaic/chimaeric MHC expression has a differential influence on reactivity towards normal vs. tumour cells, with respect to allele specific as well as general missing-self rejection.

Afterwards, aims will be revisited and discussed in Concluding remarks and future perspectives (Section 4.10).

3 MATERIALS, EXPERIMENTAL TECHNIQUES AND METHODOLOGICAL CONSIDERATIONS

The studies include immunological methods performed in experimental mouse models. Mixed bone marrow chimaeric mice as well as several transgenic mouse strains have been generated and used. Key methods used are multi parameter flow cytometry and *in vivo* assays such as target rejection assays, antibody blockade and bone marrow transplantation models. These murine models and experimental methods will be described in the following sections.

3.1 MICE

All mice were kept and bred under specific pathogen free conditions in the animal facility at the department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Solna Campus. 6-17 weeks old mice were used for experiments. Mice were age-matched for all experiments and age and gender-matched for bone marrow chimaeras. All experiments were performed according to ethical guidelines and were approved by Stockholms Norra djurförsöksetiska nämnd.

C57BL/6 mice: Commonly denoted as B6 mice, they were obtained from the breeding unit at MTC, or purchased from Taconic Europe (Lille Skensved, Denmark). These mice have an MHC-I type of K^b D^b.

B6 $\beta_2m^{-/-}$ mice: They lack the gene for β_2m component of the MHC-I resulting in none or low expression of MHC-I on the cell surface [89].

DL6 mice: The DL6 mouse carries a fusion transgene $\alpha1/2D^d$+$\alpha3L^d$ (induced via a vector construct pg24 [213]) on a B6 background, where it is expressed in a binary mosaic fashion i.e. varying fractions of cells are either positive or negative for the transgene. This is the main strain that has been generated and used for experiments performed for this study project. We have used this strain in homozygous as well as hemizygous state on a B6 background. We also generated a Ly5.1 variant for experimental purposes, as explained later. DL6 mouse is described in detail in the results section [92].

DL1 mice: This mouse, as described earlier [92], has the same fusion transgene (D^d+L^d) as the DL6 mouse, but with a global expression of the transgene, i.e. it is expressed on all cells, as opposed to the binary mosaic expression pattern in DL6 mice. Thus, it expresses an MHC-I profile of K^b, D^b and D^d molecules like the D8 mouse and both these strains have been used as controls for DL6 mice, during experiments. DL1 mice have been bred as backcrosses to B6 mice. By immunophenotyping for D^d, we determine the offsprings positive for the transgene, and use these mice for experiments and breeding.

D8 (D8 Ly5.1) mice: The D8 mouse has been already described [214]. This mouse strain has an additional complete D^d transgene (α1, 2, 3) on a B6 background and, therefore, expresses an MHC-I profile of K^b, D^b and D^d molecules. D8 mice have been used as control mice for DL6 experiments and chimaera experiments. They have also been used as donors and recipients for the mixed bone marrow chimaera and DL6 chimaera experiments, where a Ly5.1 variant, D8 Ly5.1 is also used to track cells from two different origins; donor and recipient.

B6 Ly5.1 congenic mice: They were obtained from Charles River Laboratories, Lyon, France. We backcrossed most of the mouse strains used, with B6 Ly5.1 mice [215], and then intercrossed them to get the transgene and the Ly5.1 allele in the same mouse (ensured by one or more of the following methods, as applicable; blood immunophenotyping, genotyping, ear biopsies and test crosses). This was used for the purpose of tracking donor derived bone marrow cells in bone marrow chimaeras, generated as described below.

3.2 BONE MARROW CHIMAERAS

3.2.1 Mixed bone marrow chimaeras

These chimaeras were generated by transplanting D8 and B6 donor bone marrow into D8 recipients. Prior to transplant of bone marrow cells, NK cell depletion was carried out in the donors and recipients to prevent NK cell mediated rejection. NK cell-depleting antibodies i.e. anti-NK1.1mAb (PK136) and anti-Tmβ1 mAb (anti- CD122) were tested in pilot chimaeras with respect to dose, frequency and interval of depletion. It was noted that haematopoietic reconstitution of NK cells was better in recipients treated with anti-Tmβ1 antibody, as compared to anti-NK1.1 antibody, which is an effective NK cell depleting antibody (perhaps due to a longer half-life of the latter antibody). Therefore, we decided that the donors and recipients were depleted of NK cells using different antibodies, the donors with anti-NK1.1mAb (PK136 200μg Mabtech, Nacka, Sweden) and recipients with anti-Tmβ1 mAb 100μg, Mabtech, Nacka, Sweden). Depletion was performed 3-5 days before irradiation. Bone marrow cells were obtained from the hind limbs of NK depleted donor mice. The soft tissues were removed and the tibia, femur and ilium (pelvic bone) crushed and ground in PBS with EDTA, using a mortar and pestle. PBS was used to rinse the pestle and after filtering it through a 40μm mesh, filtrate was washes and re-suspended. T cell depletion was performed by negative selection using MACS anti-CD5 beads, MACS LD columns and MACS Midi magnets (Miltenyi, Bergisch Gladbach, Germany). T cell depleted bone marrow from different mouse strains was mixed to certain ratios (ratios between 50/50 and 25/75). The recipient mice received ^{137}Cs irradiation split into 2 doses i.e. 500 and 600 rad, 4 hours before transplant. Each mouse received $10x10^{6}$ bone marrow cells via an intravenous injection in the tail vein. Mice received antibiotics (Trimethoprim Sulfadazine 0.2/1mg/ml) in drinking water for 3-5 weeks. Reconstitution was checked by immunophenotyping blood at three time points; early (day 7-11), middle (day 30-35), late (day 48-55) to assess the kinetics of haematopoietic reconstitution. Mice were used in experiments 8-10 weeks post-transplant, based on our unpublished results showing that NK cells become mature and functional around 8 weeks after transplant.

3.2.2 Bone marrow chimaeras in MHC-I mosaic recipients

These chimaeras were generated by inoculating D8 donor bone marrow into DL6 mosaic recipients. The protocol was the same as described for the mixed bone marrow chimaeras, but with some differences. No NK cell depletion of recipients was done, as the MHC phenotype of the donor cells was already present in the recipients and NK cells were tolerant. Neither were donor NK cells depleted, since they could eliminate the remaining recipient D^{d-} haematopoietic cells, thus allowing for better reconstitution. The donor bone marrow was not depleted of T cells either, as there were no additional MHC molecules in the recipients, both

donors and recipients were of the same gender and we did not expect any T cell reactivity. Thus, rejection was not expected in any direction, in this MHC-I setup. Since every mouse group received only one kind of bone marrow, these are not mixed bone marrow chimaeras.

3.3 TUMOUR CELL LINES

Tumour cell lines were cultured *in vitro* or grown as ascites *in vivo*, depending upon the amount of cells required (culture conditions- 7% CO_2 at 37^oC, in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100U/ml Penicillin, 100mg/ml Streptomycin, 2mM L-glutamine, 50mM 2ME, 1mM sodium pyruvate, non-essential amino acids). For *in vivo* growth, suitable mice were selected to avoid any rejection of the tumour cells, and irradiated sublethally (4 Gy). Frozen tumour cell lines were thawed, inoculated intraperitoneally in mice and grown as ascites for about a week. Mice were then assessed by abdominal palpation for ascites growth, and sacrificed after around 7 days. Peritoneal lavage was performed with PBS and on average ~500 x10^6 cells were recovered from a mouse. The lavage was filtered and washed with PBS to reduce coagulates and cell lysis by enzymes. Ascites has been used instead of *in vitro* cell cultures because here the cells grow in more physiological conditions and there is no cell activation that is otherwise induced in the cell cultures by the use of additives e.g. growth factors required for the cultures to maintain growth. Also, the cell yield is more in ascites, which is a requirement for *in vivo* challenge assays.

RMA: RMA (H-2^b) is a sub-line of Raucher virus-induced EL-4 T cell lymphoma of B6 origin. [78] [216]

RMA-S: RMA-S is a TAP-2 deficient variant of RMA [217]

RBL-5D^d: RBL-5D^d is EL-4 transfected with the vector pD^d-1 encoding genomic H-2D^d [218]. RBL-5 and EL-4 are identical, where RBL-5, RMA and RMA-S are all derived from EL-4.

C1498: It is a mouse H-2^b NKT tumour cell line [219], and was a kind gift from Andrew Makrigiannis, Dalhousie University, Canada. $C1498^-$ is a MHC-I^- variant of the original MHC-I^+ tumour. [74].

3.4 IMMUNOPHENOTYPING

3.4.1 Preparation of cells

Preparation of cell suspensions from spleens for immunophenotyping:

Spleens were mashed in petri dishes using plungers. The cells in the petri dishes was rinsed out with PBS and thereafter pelleted in 15 ml falcon tubes. Erythrocyte lysis was performed with either ER lysis buffer (ACK, containing NH_4Cl) and stopped by the addition of 10 times the volume of PBS, or with ddH_2O and stopped with 10x PBS. Lysate was filtered through 40μm nylon mesh strainers and after washing, re suspended in PBS. 1-1.5x10^6 spleen cells were added per well in a 96-well v-bottomed plate. After centrifuging to pellets, staining solutions were directly added to cell pellets and mixed.

Preparation of cell suspensions from blood for immunophenotyping:
100µl of heparinized blood was added per well in a 96-well v-bottomed plate. After pelleting, 200µl of BD lyse buffer (BD Biosciences, New Jersey, USA) diluted 10 times in ddH_20 was added, and samples incubated at room temperature for 20 minutes. This step was repeated once more and blood was pelleted by centrifugation. Staining solutions were added directly to pellets and mixed.

3.4.2 Cell surface staining

Cells were stained with titrated concentrations of antibodies diluted in PBS (sometimes in the presence of 0.5% FBS) and incubated in the fridge (4^oC) or on ice, usually for 30 minutes. This was followed by washing steps and resuspension in PBS. Sequentially, Fc receptors were blocked using a blocking antibody anti-CD16/32 (2.4G2), followed by surface staining. Cells were either unfixed and analysed within 4 hours on the same day, or fixed (in 1% PFA) and analysed the next day.

3.4.2.1 Typing of mice

The DL6 mice were typed before planning experiments to find out the percentages of D^{d+} fraction in each mouse. Blood was immunophenotyped using an antibody against the D^d (α1, 2) domains of the fusion transgene D^d+L^d (34-5-8S PE-conjugated, BD Biosciences, New Jersey, USA.). Blood from mice with complete D^d expression (D8, DL1) or no D^d expression (B6, $\beta_2m^{-/-}$) was used as control in the experiment. The method was same as for surface staining.

3.4.2.2 Multi-colour panels

These were designed using multiple antibody combinations to study NK cell features like receptor repertoire, maturation subsets etc. Care was taken to properly control for variables e.g. fluorochrome instability, dead cell exclusion, doublet exclusion, fluorescence minus one controls, fidelity controls, etc., along with compensation controls.

3.4.3 Flow cytometry

FACS Scan, FACS Calibur and LSRII SORP (Becton Dickinson, New Jersey, USA) flow cytometers were used. Cells were acquired using Cell Quest or Diva (BD Biosciences) softwares, at 3,000-10, 000 events/ second, depending on the machine and assay. Analyses were done using Cell Quest Pro or Flowjo (9.6, 10 Tree Star, Oregon, USA) softwares.

3.4.4 Antibodies

The following antibodies were used in flow cytometry.

From Biolegend, San Diego, USA

anti-NK1.1 (PK136, mouse IgG2)
anti-D^d (34-5-8S)
anti-CD3e (145-2C11)
anti-NKp46 (29A1.4)
anti-CD49b (DX5)

anti-Ly49A (YEI/48 A1)
anti-Ly49G2 (4D11)
anti-KLRG1 (2F1/KLRG1)
anti-2B4 (m2b4 (B6) 458.1)
anti-CD69 (HI.2F3)
anti-KLRG1 (2F1/KLRG1)
anti- CD107a (ID4B-RUO)
anti-IFN-γ (XMG1.2)
anti-CD45.1 (A20)
anti-CD45.2 (104)

From R&D systems, MN, USA.

anti-Ly49D (4E5)
anti-Ly49I ((YLI-90)
anti-NKG2D (CX5)
anti-CD27 (LG.3A10)
anti-CD11b/Mac-1 (M1/70.15)
anti-CD49b (DX5)
anti-Ly49C/I (5E6)

From BD Biosciences, New Jersey, USA

anti-Fcγ RII/III receptor blocking antibody (anti CD16/32, 2.4G2)
anti-H2-D^d-PE (34-5-8S)

Streptavidin-QD605 (Invitrogen Molecular Probes)
Live/Dead Fixable Aqua Dead cell stain kit (Molecular Probes, Invitrogen, Oregon, USA)
anti-IgG 3 anti-mouse IgG3 (Southern Biotechnology, Alabama, USA)
anti-NKG2A/C/E (20d5) (AbD Serotec, Oxford, UK)
anti-Ly49C (4LO3311 hybridoma) (kind gift from Suzanne Lemieux, Institut Armand-Frappier, Quebec, Canada)

3.5 *IN VIVO* ASSAYS

3.5.1 Spleen and tumour cell challenge assays

3.5.1.1 Two cell assay/CFSE assay

Cells were labelled with two concentrations of the fluorescent dye Carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Invitrogen, Oregon, USA.). One cell type was the internal control, expected to be retained by all recipient mice and the second cell type was the test (which we were interested in studying rejection of). Cell suspensions were prepared from spleens (normal cells)/ascites (tumour cells), and counted, as described above, under sterile conditions. The CFSE stock solution (10µM) was thawed in dark and two labelling concentrations i.e. 5µM (usually for test cells) and 0.5µM (usually for control cells) were prepared in PBS. Cells were carefully mixed with the corresponding labelling solution to

ensure a uniform labelling intensity. Labelled cells were incubated at 37°C for 10 minutes and then washed with 10 times the volume of ice cold PBS with 20% FBS (foetal bovine serum). After filtration, cells were counted and re-suspended in PBS (at a final concentration of $200x10^6$ cells/ml in case of spleen cells and $300x10^6$ cells/ml in case of tumour cells. Equal volumes of the two labelled cell solutions were mixed. Finally, 0.1ml of the solution mixture (containing $20x10^6$ spleen cells/$30x10^6$ tumour cells) was injected intravenously in the tail vein of each mouse. Some of the mixture was analysed by flow cytometry to get an inoculated ratio between the two labelled cell types. For kinetic analysis in case of spleen cell assay, blood was drawn on days 1, 3 and 4 or on days 2 and 4. On day 4 the mice were sacrificed and spleens were also analysed by flow cytometry for a final readout. For tumour cell assay, no blood was drawn and only the spleens were analysed 8-36 hours after injection (as indicated). The final ratio between the two labelled cell types was corrected for the inoculated ratio. The results were expressed as percent labelled test cells out of total cells in the spleen, or as ratio of survival of the test cells corrected for the inoculated ratio, using the following formula.

relative survival of test cells = (% remaining test cells/% remaining control cells)/ (% test cells in inoculate/% control cells in inoculate)

3.5.1.2 Three cell assay/CFSE+Violet assay

This method was developed during this thesis work. It is a modification of the two cell/CFSE assay, where one control cell and two test cell populations were inoculated and studied *in vivo*. The additional test cell type was labelled with the cell tracking dye BMQC Violet (Molecular Probes, Invitrogen, Oregon, USA). Staining concentration was determined as 10µM by titration experiments as described in results section. To accommodate 3 cell populations, the cells were re-suspended at a final concentration of about $300x10^6$ cells/ml in case of spleen cells and $450x10^6$ cells/ml in case of tumour cells. Finally, 0.1ml of the solution mixture (containing approximately $30x10^6$ spleen cells/$45x10^6$ tumour cells) was injected intravenously per mouse and the readout was done as described above but with 2 control/test cell ratios in this case.

3.5.2 NK receptor blocking assays

Intra-peritoneal inoculation of 5E6 $F(ab')^2$ fragments specific for Ly49C/I (Novo Nordisk, Denmark) was done to block self-specific inhibitory NK cell receptors. After 16 hours-7 days, mice were challenged by intravenous inoculation of fluorescently labelled $\beta_2m^{-/-}$ spleen cells and RMA lymphoma cells, and measurement of rejection was done. The readout for the two independent cell types was shown as percent labelled test cells out of total cells in the spleen.

3.5.3 Intra-peritoneal assay

$C1498^+$ ($MHC\text{-}I^+$) and $C1498^-$ ($MHC\text{-}I^-$) tumour ascites were obtained from mice. CFSE labelled tumour cells were mixed with NK1.1 depleted BMQC Violet labelled syngeneic cells from impaired missing-self recognition (IMSR) mice [220] as reference cells, and

inoculated intraperitoneally as a mixture. Readout was done 6 hours later in spleens. Naïve, NK depleted and Poly I:C (Sigma, Missouri, USA) activated mouse groups were used.

3.6 STATISTICS

Data from flow cytometry were obtained and plotted using Microsoft Excel and GraphPad prism 6.0 (California, USA). Results were shown as data for individual mice or as group means with standard deviation. Dunnett's test for multiple comparison was applied when comparing different groups. The p-value of less than 0.05 was considered as significant. Pearson's correlation coefficient or r was also determined for measuring strength of linear associations.

4 RESULTS AND DISCUSSION

4.1 PERTURBED MHC-I SENSING BY NK CELL INHIBITORY RECEPTOR BLOCKADE

Studies have shown that adoptive transfer of activated NK cells with missing-self activity towards the host can, in certain settings, have a corresponding beneficial anti-leukaemic effect [154] [221]. In previous studies by our group and others (reviewed in the introduction), it has been explored whether the same beneficial anti-tumour effect can be achieved in an autologous situation where MHC-I sensing is perturbed. In the study by Vahlne et al. [168], an *in vivo* scenario mimicking missing-self recognition was achieved by antibody mediated blockade of certain NK inhibitory receptors. The Ly49C/I receptors expressed by 34-60% of NK cells in the B6 strain, were targeted using F(ab')^2 fragments of a blocking antibody from the hybridoma 5E6. Upon challenge with fluorescently labelled MHC-I sufficient spleen cells and tumour cells (RMA lymphoma cells), the tumour cells were more efficiently eliminated by NK cells of the antibody treated mice, while self tolerance to spleen cells was maintained. No signs of autoimmunity were observed.

Considering the importance of self MHC-I-inhibitory receptor interactions in NK cell education (as discussed in introduction), the same setting was explored to study the functional maturity of developing NK cells and the retuning potential of mature NK cells. We reasoned that the robust tolerance to normal cells might be explained if one assumes that prolonged *in vivo* blockade of inhibitory receptors leads to a re-adjustment of NK cell activation threshold in order to avoid autoreactivity, in line with the predictions of the "rheostat model". An implication of this hypothesis and reasoning was that NK reactivity to *normal* cells representing missing self should be *decreased* by inhibitory receptor blockade, even if the killing of MHC-I expressing tumour cells was increased. We tested this prediction in studies led by Stina Wickström, a colleague in the group. This project was part of a bigger study, in which another approach to test possible "retuning" effects was also used: adoptive transfer of NK cells between mice with different MHC-I genes [222].

In the studies of antibody blockade, we were particularly interested in testing the kinetics of possible retuning effects, as measured by reduced rejection of MHC-I deficient, but otherwise normal spleen cells. Different groups of B6 mice were given intra-peritoneal inoculation of 5E6 anti-Ly49C/I F(ab')^2 (at various time points) and then challenged by intravenous inoculation of fluorescence labelled syngeneic B6 spleen cells, β_2m deficient spleen cells and MHC-I expressing syngeneic tumour cells (RMA lymphoma). We measured the survival of each of these target cells in parallel in a short term assay. We reasoned that two different effects of inhibitory receptor blockade might have distinct kinetics: the blockade in the effector/target interaction resulting in increased killing of MHC-I$^+$ lymphoma cells would occur rapidly within hours, while the retuning effect would take longer, perhaps several days.

The experiments showed that while the inhibitory receptor blockade with 5E6 anti-Ly49C/I F(ab')^2 increased *in vivo* NK mediated killing of MHC-I expressing lymphoma cells (Figure 4; [168]), it did indeed reduce the killing of β_2m deficient, but otherwise normal spleen cells, as predicted by the hypothesis. In contrast, in mice treated with a control antibody binding a non-self specific inhibitory receptor (4D11 binding Ly49G2), killing of β_2m deficient spleen

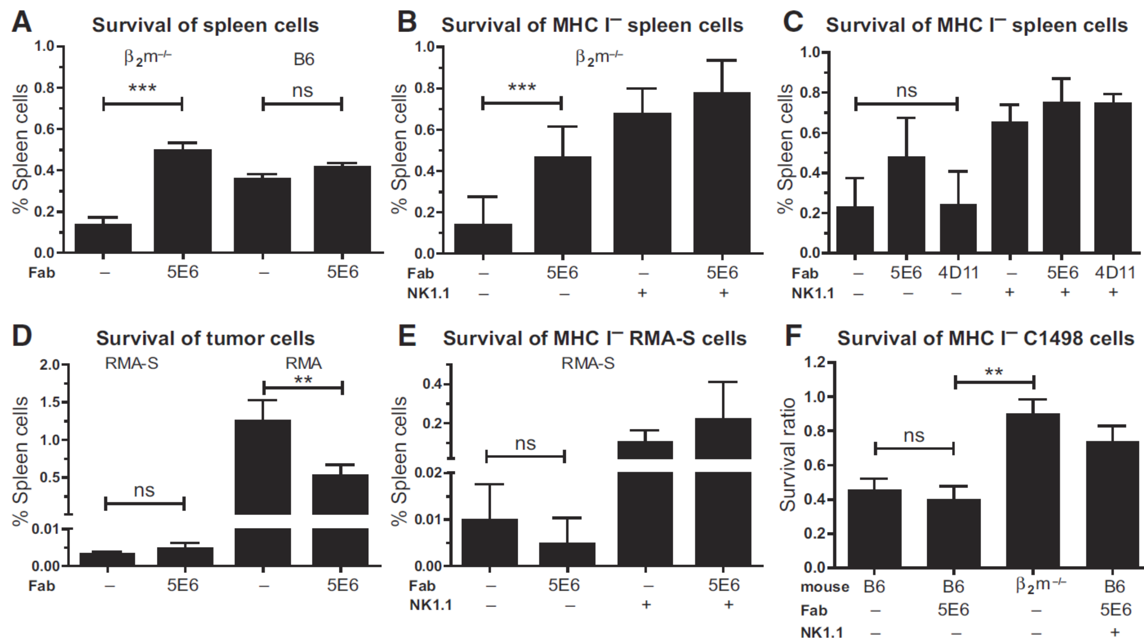


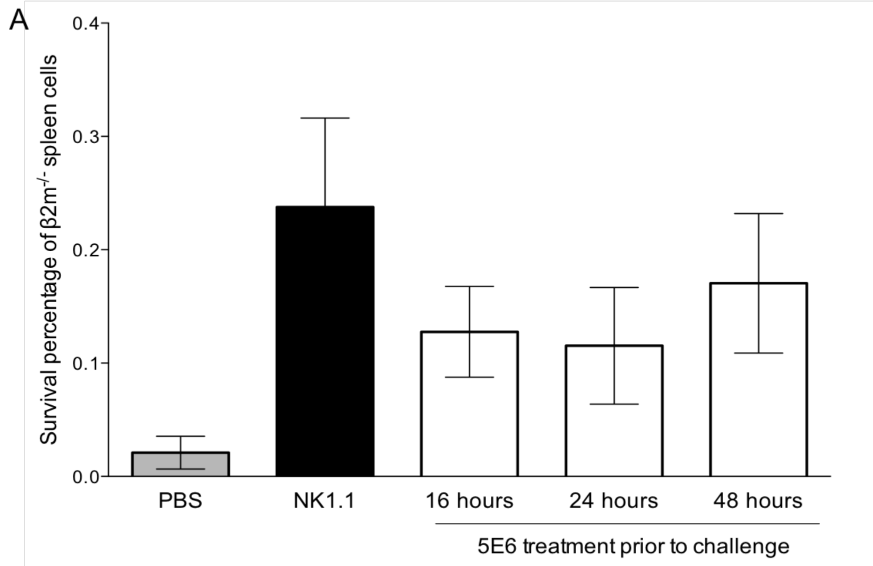
Figure 4. Inhibitory receptor blockade reduces rejection of normal cells, but not tumour cells. A–F, CFSE-labelled cells were inoculated i.v. into B6 mice that were untreated or treated with the indicated blocking reagent alone or in combination with depleting anti-NK1.1 mAb. Results are displayed as mean surviving target cells in percentage of total spleen cells (A–E) or as a ratio of target cells versus control cells (F). A–E, 5E6 (F(ab')2 binding to Ly49C/I) or 4D11 (F(ab')2 binding to Ly49G2) with or without anti-NK1.1 mAb was administered i.p. 48 hours before CFSE-labelled B6 (A), ß2m$^{-/-}$ (A and B), RMA (D), or RMA-S (D and E). A–E, cell suspensions were inoculated i.v. and survival of target cells was analysed 44 hours (A–C) or 24 hours (D and E) later in the spleen. F, C1498-MHC^{-} cell suspension was coinjected i.p. with MHC I^{+} NK cell-depleted control spleen cells. Six hours later, survival of target cells was analysed by peritoneal lavage. The survival ratio of the parental MHC I^{+} C1498 line was 1.37±0.5 in B6 and 1.68 ± 0.8 in NK1.1-depleted B6 mice (P = 0.4977). A shows one representative experiment out of five, with 4 mice per group, B shows a compilation of five experiments with a total of at least 17 mice per group, and C shows a compilation of three experiments with at least 11 mice per group except the NK1.1-treated group, which had 6 mice in two experiments. D shows 4 mice per group, one representative out of two independent experiments, E shows a compilation of two experiments with a total of 8 mice per group, F shows three experiments with 8 mice per group except for the NK1.1-treated group, which has 4 mice. Error bars, SD. Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant. (Reproduced with the permission of the publisher).

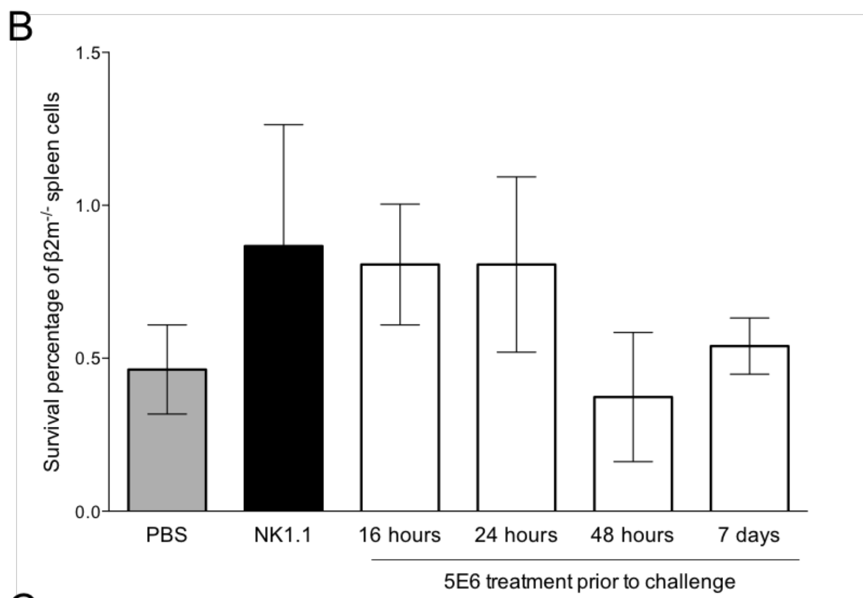
cells was unaffected (Figure 4A-C). MHC-I expressing spleen cells were not killed *in vivo* by NK cells, regardless of whether the mice had been treated with 5E6 anti Ly49C/I F(ab')^2 or not (Figure 4A). Killing of MHC-I deficient tumour cells was, however, maintained, as examined by the challenge with two independent tumour cell lines: RMA-S and C1498 MHC^{-} (Figure 4D-F). Our interpretation is that the reduced killing of MHC-I deficient spleen cells reflects a "retuning" imposed by the reduced inhibitory input after receptor blockade. We further speculate that this retuning would maintain tolerance to normal cells, but would still allow killing of tumour cells due to their higher expression of ligands for activating NK cell receptors.

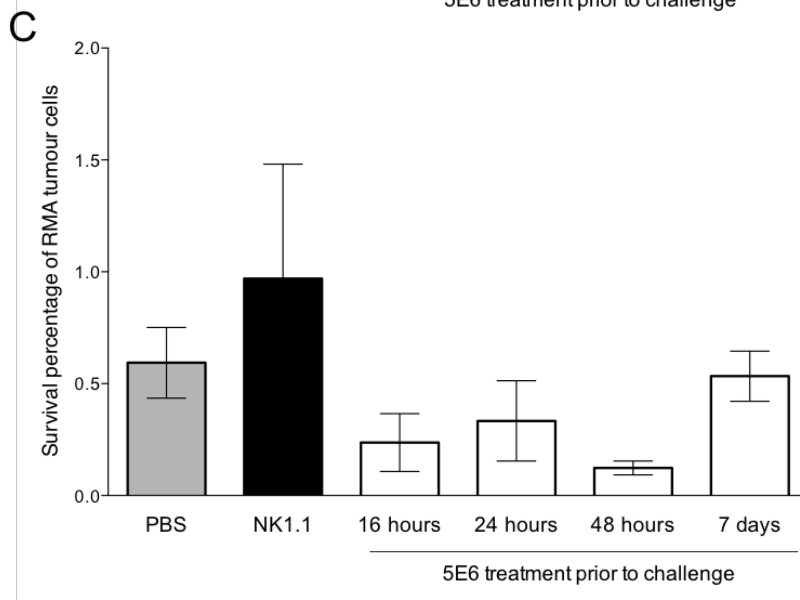
Surprisingly, the induction of reduced killing of normal MHC-I deficient cells did not require several days; it was observed reproducibly within 24 hours and often as early as after 16 hours of receptor blockade, indicating that the retuning is a fairly rapid process (Figure 5). Despite several attempts using the two targets in the same mice, we were not able to distinguish a reproducible kinetic difference between this process and the one resulting from the action of inhibitory receptor blockade in the effector target interaction leading to increased killing of MHC-I^{+} lymphoma cells (Figure 5). One reason for this may be the poor time scale resolution of this type of assay, which uses 24-48 hours to monitor the end point effect of retuning, i.e. *in vivo* killing of normal cells. Future studies should address the kinetics of retuning using a shorter assay allowing also specific monitoring of the targeted NK cell subset (Ly49I and Ly49C expressing NK cells), e.g. the *in vitro* single NK cell responsiveness assay measuring degranulation (CD107α) and cytokine production (IFN-γ) after stimulation with plate bound antibodies against activating NK cell receptors. In our published study [222], it was also demonstrated that the Ly49I (but not the Ly49C) single positive NK cells were indeed rendered hyporesponsive by the antibody blockade, but the kinetics of this effect were not explored.

In these experiments, we could thus confirm that NK cell education for missing self rejection can be influenced rapidly by perturbed sensing of MHC-I in the host environment. The results are highly relevant to similar NK inhibitory receptor blockade in clinical trials, because although we observed a beneficial effect on tumour cell killing, it cannot be excluded that this was a net effect that would have been more impressive if it had not been influenced by a general retuning of NK cell responsiveness. It has indeed been recently reported by Carlsten et al. that also in humans, antibody-mediated blockade of inhibitory receptors (KIR2D) leads to hyporesponsiveness of targeted NK cells, demonstrated by the observation that KIR2D single positive NK cells showed a reduced responsiveness to K562 tumour cells *in vitro* after receptor blockade. Furthermore, in this clinical phase II trial including nine patients with smouldering multiple myeloma, no clinical benefit of the treatment could be demonstrated [223]. In future studies of inhibitory receptor blockade, it will be important to explore different doses and administration regimens of the blocking antibody, in order to optimize the effects on the effector-tumour target interaction, and minimize the retuning effect that presumably occurs during continuous interactions between NK cells and normal cells.

The experimental situation tested in the studies above involved reduced general sensing of MHC-I on all cells in the host, at least by one particular NK cell subset. However, the results have implications also for other clinical situations where MHC-I sensing is perturbed in a fraction of the cells in the host environment, i.e. in situations of host/donor chimaerism. We reasoned that such a heterogenetic environment may have similar effects on NK cell education and tolerance, and in particular, that influence may depend on the degree of chimaerism. We thus set out to investigate quantitative aspects of education for missing-self recognition in different models for MHC-I chimaerism/mosaicism.


A
Survival percentage of $\beta 2m^{-/-}$ spleen cells
0.4
0.3
0.2
0.1
0.0
PBS
NK1.1
16 hours
24 hours
48 hours
5E6 treatment prior to challenge


B
Survival percentage of $\beta 2m^{-/-}$ spleen cells
1.5
1.0
0.5
0.0
PBS
NK1.1
16 hours
24 hours
48 hours
7 days
5E6 treatment prior to challenge


C
Survival percentage of RMA tumour cells
2.0
1.5
1.0
0.5
0.0
PBS
NK1.1
16 hours
24 hours
48 hours
7 days
5E6 treatment prior to challenge

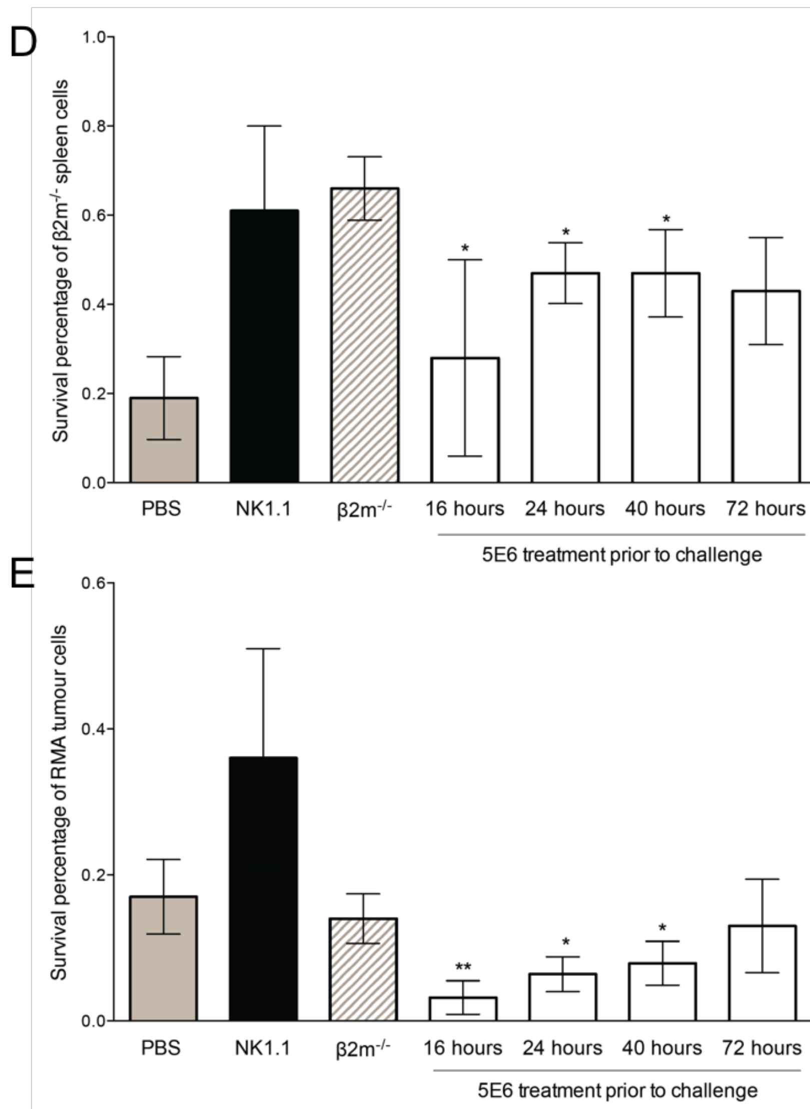


Figure 5. Kinetic analysis of effects on rejection capacity after inhibitory receptor blockade. A-E. CFSE-labelled cells were inoculated i.v. into B6 mice that were untreated, treated with 5E6 blocking reagent or with depleting anti-NK1.1 mAb (A-E), or ß2m$^{-/-}$ control mice (D-E). Results are displayed as mean surviving target cells in percentage of total spleen cells. 5E6 F(ab')2 was administered i.p. at indicated time points before CFSE-labelled ß2m$^{-/-}$ (A, B and D) or RMA (C and E) cell suspensions were inoculated i.v. and survival of target cells was analysed 48 hours (A-C) or 44 hours (D-E) later in the spleen. Three individual experiments are shown (A, B-C and D-E respectively) with 3 mice per group. Error bars, SD. Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$ (D-E).

4.2 CHARACTERISTICS OF DL6 MICE WITH MOSAIC EXPRESSION OF AN MHC-I GENE

4.2.1 Previous studies of DL6 mice

DL6 mice were briefly introduced above as the first model to provide evidence for reversible education of NK cells. These mice show a mosaic MHC I transgene expression, with cells of two different phenotypes: $K^bD^bL^d/D^d$ and K^bD^b. These mice were identified in a transgenic line on B6 background (providing K^b and D^b genes), into which an engineered D^d gene (with α3 domain from L^d and α1/α2 domains from D^d) had been introduced. This L^d/D^d chimaeric MHC-I gene product functioned as the wild type D^d gene with respect to recognition by NK cell inhibitory receptors, as shown with transfected target cells [224-226] and also with another transgenic line on B6 background, (DL1), where the transgene was expressed as a normal MHC-I gene, i.e. in all cells [225]. In DL1 mice as well as in D8 mice (wild type D^d transgene on B6 background), NK cells recognized cells lacking D^d as “missing self” [92, 218, 227, 228]. For simplicity, this chimaeric gene used to generate DL1 and DL6 mice will therefore be referred to as D^d in the remainder of the text. A summary of the mouse strains used in the study is shown in Table 2.

Table 2. Mouse strains used in this thesis work

Mouse strain	MHC-I transgene	Transgene expression	Endogenous MHC-I genotype	MHC-I α1/α2 phenotype	Phenotype designation
B6*	N.A.	N.A.	K^b D^b	K^b D^b	D^{d-}
$β_2m^{-/-}$	N.A.	N.A.	K^b D^b	K^{b-} D^{b-}	$β_2m^{-/-}$
D8*	D^d	all cells	K^b D^b	K^b D^b D^d	D^{d+}
DL1	$α1/α2D^d$ / $α3L^d$	all cells	K^b D^b	K^b D^b D^d	D^{d+}
DL6*	$α1/α2D^d$ / $α3L^d$	mosaic	K^b D^b	K^b D^b D^d & K^b D^b	D^{d+} & D^{d-}

* Ly5.1 congenic variants of these strains were used as well.

Johansson et al. studied how the mosaic MHC-I expression in DL6 mice affects the education of NK cells, in particularly whether cells lacking the D^d ligand would be recognized as “missing self” also in these mice [92]. The methodology included *in vitro* NK cytotoxicity, bone marrow transplant rejection and measurements of tumour outgrowth. These assays showed a complete tolerance against cells lacking the critical MHC-I D^d molecule in all mosaic mice. However, the tests used did not allow a quantitative analysis of rejection capacity, in particular the *in vivo* assays where the simple end point was either growth or rejection. More quantitative assays were needed in order to appreciate the kinetics and magnitude of rejection, and to get a picture of NK cell tolerance to normal cells of not only tumours but also healthy spleen cells *in vivo*, without having the need to activate NK cells priorly and artificially. This was particularly important in view of the emerging models on NK cell education and tolerance, highlighting quantitative aspects (i.e. the rheostat model discussed above), and the methods becoming available to study that, including *in vivo* cytotoxicity assays as developed in the previous section. An additional reason to revisit the DL6 model was that very few mice in the initial studies showed a proportion of D^{d+} cells

higher than 75%. We reasoned that the breaking of tolerance to missing self might require a very high proportion of such cells, and we hoped to find this if we tested large amounts of D^d homozygous DL6 mice, which had not been done in previous studies.

Offspring from breeding of homozygous DL6 mice were immunophenotyped by flow cytometric analysis of blood cells using anti-α1α2-D^d -PE antibody. All DL6 mice had a mixture of D^d positive and negative cells albeit in varying proportions. Examples of histograms from immunophenotyping of individual mice are shown in Figure 6. Non-transgenic B6 control mice showed less than 1% while D8 and DL1 showed more than 97% cells in the D^d positive gate. In earlier studies [92], it was confirmed that all mice in the backcrosses who had inherited the transgene (as determined by Southern blot) also expressed it in at least a fraction of the blood cells.

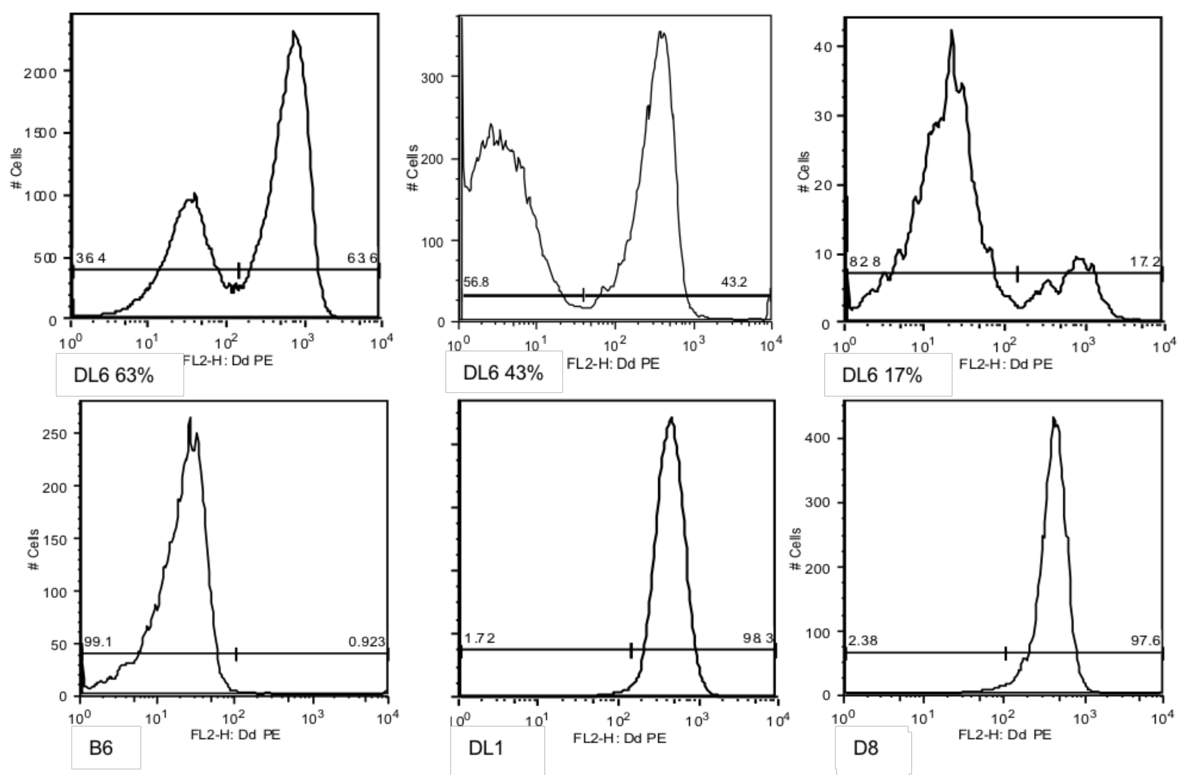


Figure 6. DL6$^{+/+}$ mice show variable frequencies of D^{d+} cells in peripheral blood. Peripheral blood lymphocytes were stained for D^d and analysed by flow cytometry. Plots show data gated on lymphocytes, from three individual DL6 mice in the top panel and control mice B6, DL1 and D8 in the bottom panel. The fraction of D^{d-} and D^{d+} cells are shown in the respective histograms.

4.2.2 D^d expression pattern in different crosses and breeding lines of DL6 mice

We typed more than 1300 mice carrying the D^d/L^d transgene in homozygous or heterozygous form in the present study. The breeding crosses performed, in general, confirmed the observations made in the earlier studies [92] concerning inheritance and expression patterns.

The transgene was thus inherited and expressed as a single (autosomal) dominant gene, i.e. all mice in F1 crosses of DL6 mice expressed the transgene and in backcrosses of such mice to B6 mice 50% expressed the transgene. The transgene was always expressed only in a fraction of the blood cells (10-80%). The proportion was roughly the same in T cells, B cells, NK cells as well as in fibroblasts. It should be noted that the latter had a low MHC-I expression in general and appeared negative for all molecules, including K^b and D^b. After exposure to IFN-γ for 48 hours, most of the fibroblast became positive for K^b and D^b while the staining pattern for the D^d transgene appeared mosaic in a similar fashion as the lymphocytes (data not shown).

The proportion of D^{d+} cells in peripheral blood remained stable within each mouse when followed over time. In the present study, we tested directly whether the mosaic expression pattern was determined at the RNA level. D^{d+} and D^{d-} cells from the spleen were separated by flow cytometry and mRNA for D^d was assessed by PCR. Only D^{d+} cells showed presence of the transcript (data not shown).

The fraction of transgene expressing cells varied between the mice within each litter along the same range as between litters. The transgene was expressed in a similar fashion in female and male mice, arguing against X chromosome inactivation as a mechanism to explain the mosaicism. Furthermore, the fraction of cells expressing the transgene was independent of the sex of the parent transmitting the transgene, the fraction of expressing cells in the transmitting parent and whether the proband was heterozygous or homozygous for the transgene (no parental imprinting).

We reasoned that a first simple possibility to identify mice with very high frequencies (>80%) would be to type many DL6 mice, thus increasing the chance to find the extremes. In the present study we typed 861 homozygous DL6 mice and observed that the frequency of D^{d+} cells varied from 8 to 93% (Figure 7). The mean frequency was 43%. Homozygous DL6 mice were crossed to B6 mice, to generate D^d hemizygous mice (169) in order to repeat, and compare to earlier DL6 studies that had been carried out on hemizygous DL6 mice. The frequencies of D^{d+} cells largely overlapped with those of homozygous DL6 mice, but there was a lower maximum frequency (78%) and a lower mean frequency of D^{d+} cells i.e. 37%.

In the previous study, the frequency of D^{d+} cells ranged between 10% and 80%. It was particularly interesting for us to identify mice with a very high frequency of D^{d+} cells in which we could study tolerance towards D^{d-} cells. To our disappointment, only a few mice had more than 90% of D^{d+} cells, as seen in the distribution in Figure 7. However, by coincidence, we obtained mice with a distribution skewed towards higher frequencies of D^{d+} cells in a cross where DL6 mice were bred to B6 Ly5.1 mice. This cross was originally made to utilize the (CD45.1/2) Ly5.1/5.2 system to track transplanted cells in bone marrow chimaeras. When we typed the Ly5.1 DL6 mice (n=261), we unexpectedly found that these showed higher frequencies of D^{d+} cells, ranging from 59% to 97% and a mean frequency of 73% (Figure 8).

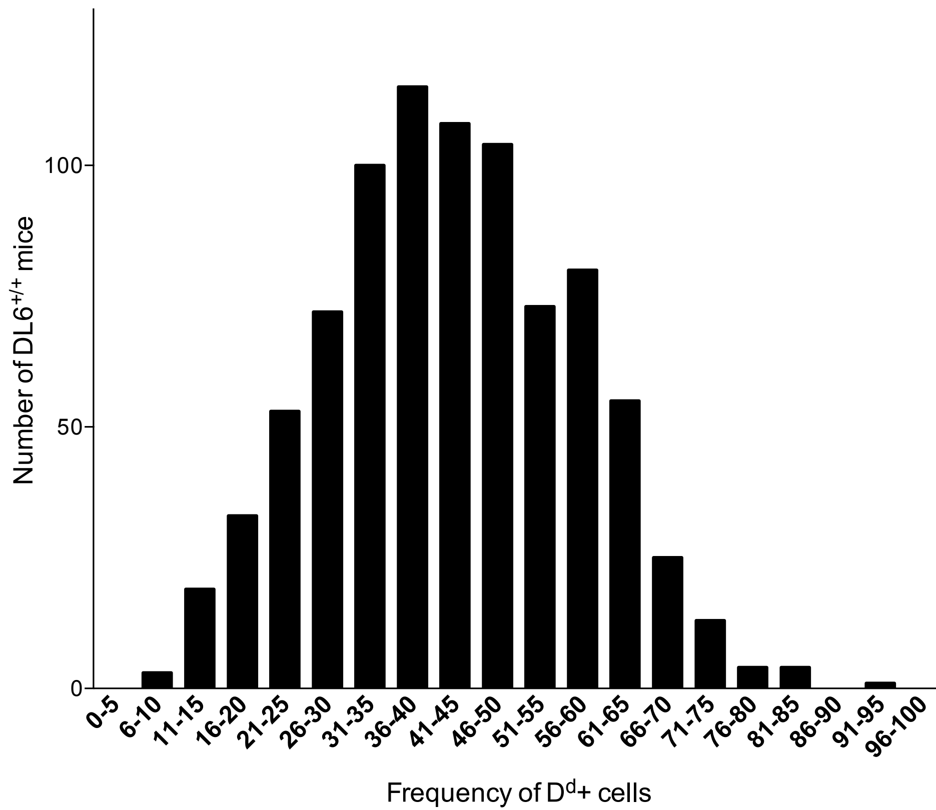


Figure 7. Distribution of frequencies of D^{d+} cells in peripheral blood of individual DL6$^{+/+}$ mice. Peripheral blood lymphocytes were stained for D^d and analysed by flow cytometry. Individual mice divided, based on frequency of D^{d+} cells, in 5% increments and shown as a histogram. n=861.

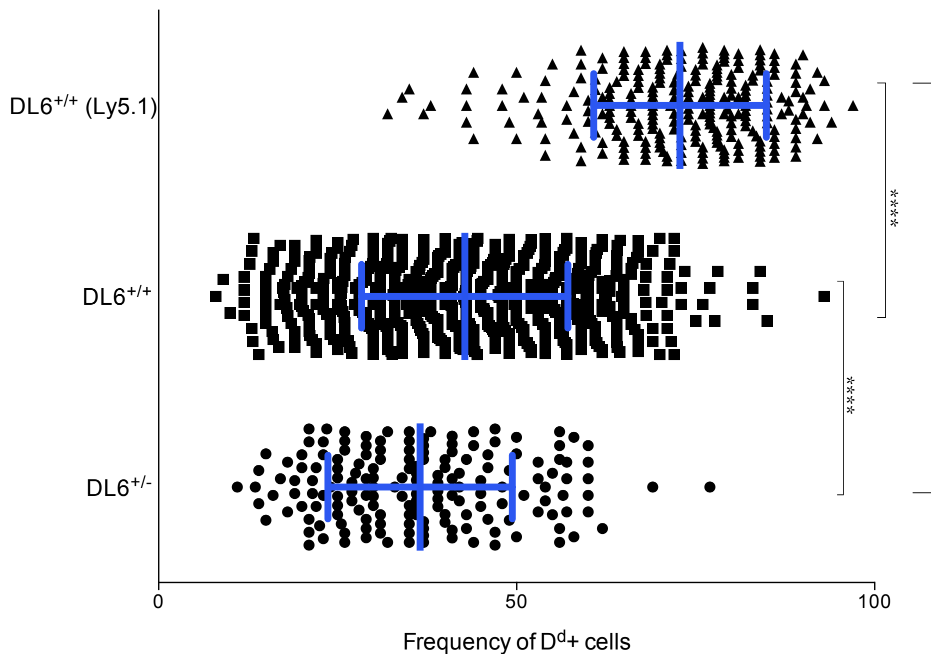


Figure 8. Distribution of frequencies of D^{d+} cells in peripheral blood of individual mice from different DL6 breedings. Data from flow cytometry analysis, figure shows DL6$^{+/+}$ (n=861), DL6$^{+/-}$ (n=169) and DL6$^{+/+}$ Ly5.1 (n=261). Error bars, SD. Statistically significant differences are denoted: ****, $P < 0.0001$

4.2.3 Cell surface expression levels of the D^d transgene

The D^d cell surface expression levels/fluorescence intensity (MFI) in D^d homozygous DL6 mice was slightly higher than that of hemizygous DL6 mice (Figure 9), showing that D^d molecule intensity of expression might be relative to mono- or bi-allelic expression of the D^d transgene. There was thus, no apparently strong gene dose effect at the level of cell surface expression, similar to observations in mice carrying the wild type D^d transgene on B6 background (i.e. where K^b and D^b is co-expressed, data not shown). In contrast, there is a clear difference in D^d cell surface expression when the D^d wild type transgene is expressed in homozygous vs. hemizygous form in the absence of any other MHC-I genes, in 'D^d single mice' [98]. The absence of a clear-cut D^d gene dose effects in DL6 mice expressing the transgene on B6 background, had one important practical consequence: it was impossible to type for mono- versus bi-allelic expression in the gene backcrosses where both of these outcomes were possible.

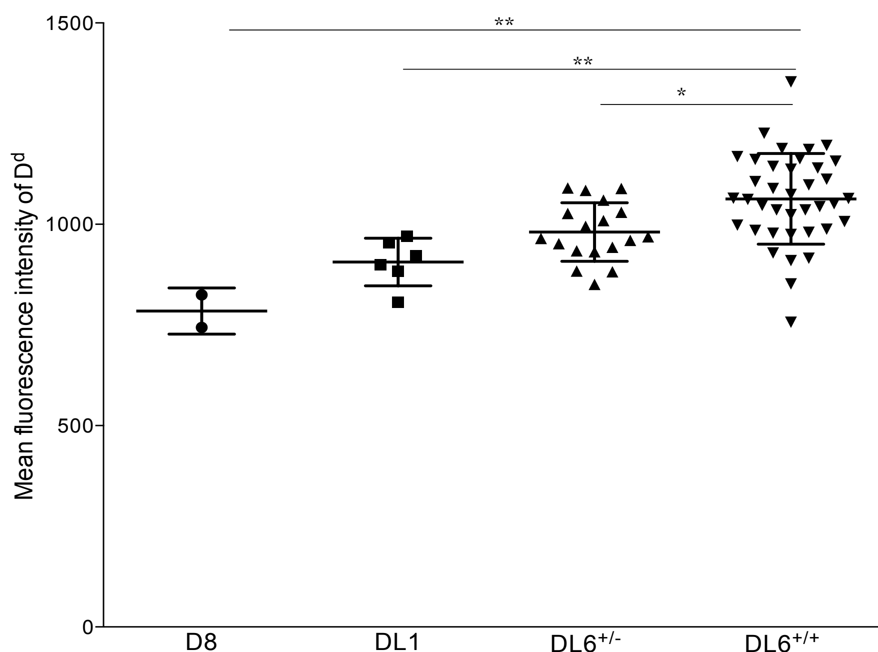


Figure 9. Cell surface expression levels of D^d in peripheral blood of individual mice from different breedings. Data from flow cytometry analysis, figure show geometric mean fluorescence intensity (MFI) of D^d staining in D8 (n=2), DL1 (n=6) $DL6^{+/-}$ (n=18), $DL6^{+/+}$ (n=38). Error bars, SD. Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$

4.2.4 Possible genetic mechanisms behind the expression patterns of the D^d transgene in DL6 mice

Mosaic or variegated gene expression patterns have been described previously, both for natural genes and transgenes [229]. It may be that this expression pattern is "under diagnosed" in transgenic settings, since mice in such studies are often typed exclusively in assays that do not allow for analysis at the single cell level. It should be noted that not only

was there mosaic expression of the transgene, the degree of mosaicism varied between the mice within the same litters of the inbred homozygous DL6 strain. The most likely interpretation for this expression pattern is that the site of integration of the transgene makes it prone to epigenetic inactivation (or activation) early during embryonic development [92]. Stochastic variations in the junctions between active and inactive chromatin may then lead to differences in expression pattern between individual mice, similar to position effect variegation described in Drosophila [230]. If this is determined in an irreversible fashion early during development, it may lead to a stable proportion of transgene expressing cells in most or all cell lineages within the same mouse, but different between mice in the same litter.

How can one then explain the distribution of frequencies of D^{d+} cells in the Ly5.1 DL6 mice? One speculation is that the flanking regions of the *Ly5.1* (CD45.1) gene (originally derived from the SJL strain [215]) contain genes coding for factors involved in regulation of transcription or epigenetic regulation. Indeed, biological differences between the congenic Ly5.1 and Ly5.2 B6 strains have been reported [231, 232]. The backcrosses to B6 Ly5.1 mice might thus have introduced a factor that influences the probability for expression of the D^d transgene, skewing the distribution towards higher frequencies of D^{d+} cells. Other possibilities are not excluded and further analyses are required.

4.2.5 Potential influence of downstream cellular interactions on the frequency of D^{d+} cells in DL6 mice

Considering all typed mice within the different breeding lines, we observed a range between 8% and 93% with respect to the frequency of D^{d+} cells. This observation imposed some practical limitations for the purpose of our study, where mice at both extreme ends were interesting. The observation also stimulated us to speculate about the implications of the absence of mice with frequencies of D^{d+} percentages below 8% and above 93%. Could this expression pattern also be influenced by events downstream of gene regulation, including immune mechanisms highly relevant to our studies?

Regarding the upper end of the range, one of the possible outcomes in mice with a higher frequency of D^{d+} cells was that NK cell tolerance to D^{d-} cells would be obliterated, leading to further shrinkage of the D^{d-} component due to NK cell mediated killing. In that case, one would observe a frequency range of D^{d+} cells approaching and capping at around 80/85%, beyond which the mice would express no D^{d-} component due to rejection. Instead, there would be a peak at the highest end of the range i.e. 100% (or just below 100%) D^{d+} cells, giving rise to a bimodal distribution. This, however, was not seen, corroborating the observation that the D^{d-} component, irrelevant of its size, maintains itself and drives tolerance/coexistence of the two cell types which are phenotypically different with respect to MHC-I.

Conversely, we speculated about the possibility that in DL6 mice with low frequency of D^{d+} cells, NK cell as well as T cell tolerance to D^{d+} cells might be obliterated. In line with the reasoning above, we would then expect the opposite bimodal distribution, with no mice showing proportions of D^{d+} cells below the critical threshold, and then higher numbers of mice with no D^{d+} cells at all. However, this was not observed, and the data thus suggest that tolerance towards D^d is maintained even when very few cells express this MHC-I molecule.

Finally, we have considered the possibility that foetal abortion of offsprings with more extreme frequencies of D^{d+} cells (positive or negative) might explain the observed distributions of expression. Such a spontaneous abortion pattern might be related to immune or other mechanisms. However, the litter sizes remain comparable to other breedings (e.g. DL1 and D8 mice, data not shown). As already mentioned, no indication of the influence of maternal frequency of D^{d+} cells on the corresponding offsprings' frequencies have been observed.

Altogether, the data thus suggest epigenetic regulation as a mechanism behind the expression patterns of the D^d gene in the DL6 mice. More studies are required to understand the regulation of this transgene, and this goes beyond the scope of this thesis.

Regardless of the mechanism, the DL6 model provided a tool to realize a quantitative way to address NK cell education, tolerance and tumour reactivity with respect to allele specific missing self as well as general missing self, in the context of normal as well as tumour cells. This required methods to study NK cell mediated rejection of several target cells *in vivo* simultaneously, which is the subject of the next section.

4.3 REFINED *IN VIVO* ASSAYS FOR NK CELL MEDIATED REJECTION OF MULTIPLE TARGET CELLS

4.3.1 Development of an assay to measure rapid *in vivo* elimination of three target cells simultaneously

One of the major uses of the assay for short term rejection of fluorescence labelled target cells (hereafter called short term rejection assay) in the context of NK cell education studies, is to assess missing self induced rejection *in vivo*, usually by comparing a test population (MHC-I deficient) to a control population (MHC-I sufficient). In the DL6 mosaic mouse, however, we have two MHC-I phenotypes, $K^bD^bD^d$ and K^bD^b, and this required a refined test. We needed to study three different target cells: one with complete 'missing-self' phenotype (MHC-I deficient, representing general missing self responsiveness), one lacking only the D^d allele (still expressing K^b and D^b, representing allele specific missing self rejection) and one control expressing the complete MHC-I phenotype, D^d along with K^b and D^b. We used spleen cell targets from MHC transgenic and knockout mice as well as tumour cells. The three tumour lines used in this study are the three genetic variants RBL5-D^d ($K^{b+}D^{b+}D^d$), RMA ($K^{b+}D^{b+}D^{d-}$) and RMA-S (with severely impaired MHC-I expression and perceived as MHC-I deficient by NK cells; as described in the materials section). Tumour ascites lines, as opposed to *in vitro* cultured tumours were used to avoid possible alterations in *in vitro* cultures that might affect rejection, and to accommodate the need for large quantities of cells needed for *in vivo* rejection studies.

We used two different dyes: CFSE together with the cell tracker dye Violet (BMQC Cell tracker). This dye enters cells and forms impermeant products. It is also toxic to cells in higher concentrations. As we were planning to use it for the spleen target cells (measured over a longer time period but with non-dividing target cells) as well as the tumour target cells (measured over a shorter time but with dividing target cells), it needed to be titrated and tested separately for the different types of cells.

In the titration experiments, we labelled syngeneic B6 spleen cells (not rejected) with different concentrations of the Violet dye. The cells were analysed by flow cytometry and inoculated in mice. Several test concentrations (0.5, 1, 5, 10, 20μM) of Violet dye were used in separate groups and inoculated cells were followed *in vivo* over 4 days (the length of our longest rejection assay) through analysis of blood or spleen cells at the end of the assay (Figure 10). We found that the Violet dye had to be used at a high concentration. Also, it was not possible to use it in more than one concentration in the same cell mixture (like we did for CFSE) as it lost intensity *in vivo,* and even at a high concentration the fluorescence intensity was low at the end of the assay. The lowest concentration of Violet dye that allowed the labelled cells to be clearly discernible from negative cells was 10μM and cells labelled with this concentration were analysed also in a mixture with $CFSE^{hi}$ and $CFSE^{lo}$ cells in order to confirm that all three cell populations could be clearly distinguished at the end of the assay (Figure 11). The same Violet concentration was found to work best also for tumour cell labelling (data not shown).

4.3.2 Kinetics of spleen and tumour cell rejection

For normal spleen cell targets, rejection was followed over four days (in blood) and finally after sacrifice (in spleens). The kinetics of the assay was analysed to identify a window where rejection could be appreciated without losing control cell populations significantly. As shown in Figures 11 and 12, sufficient resolution between cell populations and maximal rejection of all susceptible cell types were achieved on day 4. In the tumour challenge assay, rejection was more efficient and due to proliferation of target cells, fluorescence was lost more rapidly, at 36 hours the labelled populations could not be clearly detectable, rejection thus had to be measured at earlier time points (up to 24 hours) (Figure 13) and measured only in spleen. Blood analysis was not reliable due to low numbers of tumour cells in the blood (not shown).

One concern we had regarding the three cell assay was that the addition of a third cell population might affect the kinetics or extent of rejection of the other target cells. This could be due to target cell competition or target cell interaction dynamics within the inoculate. To address this issue, we conducted experiments where we compared rejection of $MHC\text{-}I^{-}$ cells using the two-cell assay and the three-cell assay (Figure 14). We found that target cells were similarly rejected in both the two-cell and the three-cell assay. This observation was of significance as the initial experiments done in the DL6 study were two-cell assays and later, we used the three-cell assays to expand the study scope, and findings from both needed to be comparable.

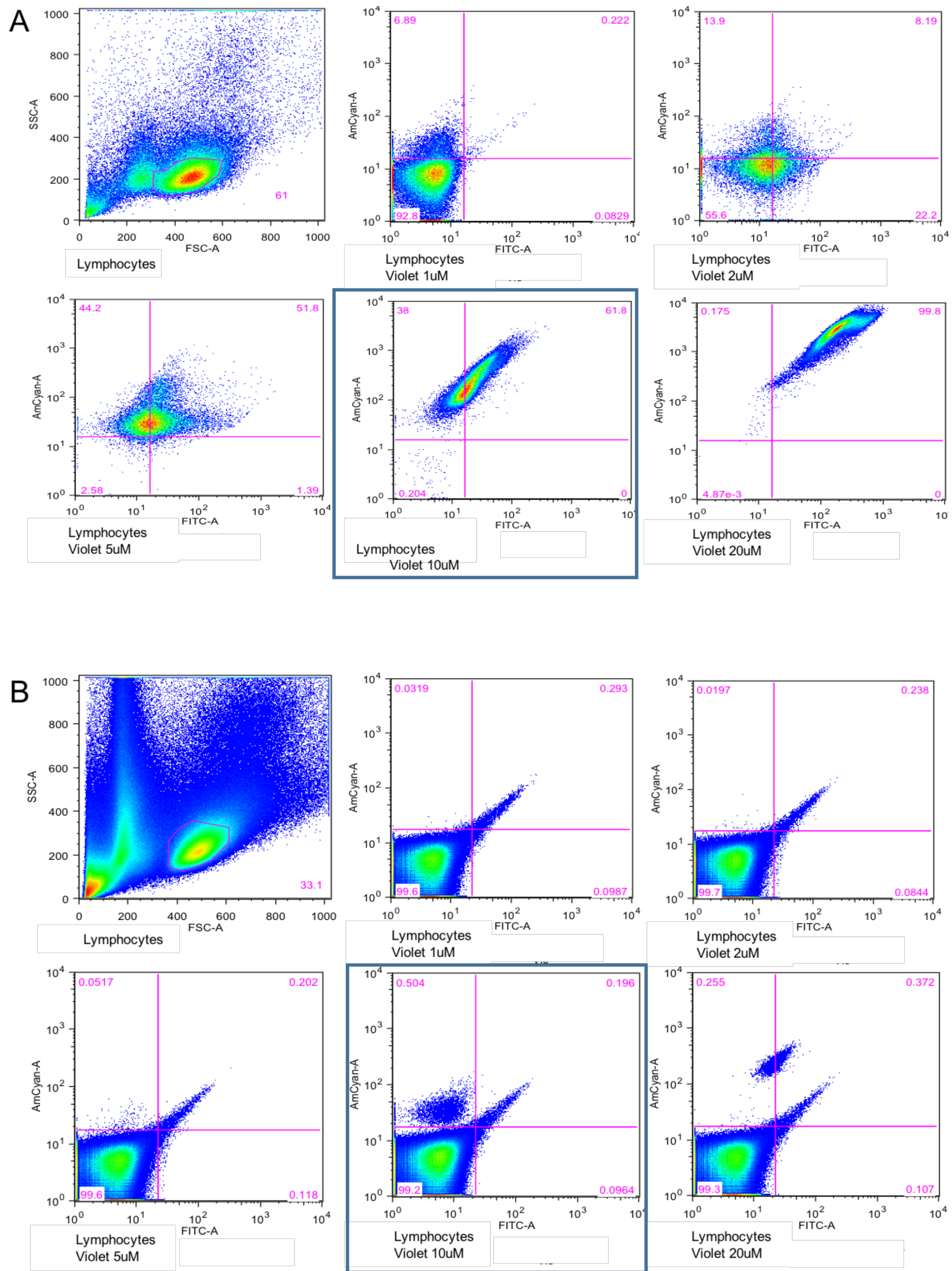


Figure 10. Titration of Violet (BMQC) dye on spleen cells used as in vivo target cells. Spleen cells were labelled with different concentration of Violet dye, inoculated i.v. into syngeneic mice and analysed in the spleen after four days. Plots show flow cytometry analysis A. directly after labelling and, B after four days. The concentration chosen for further experiments (10µm) is indicated.

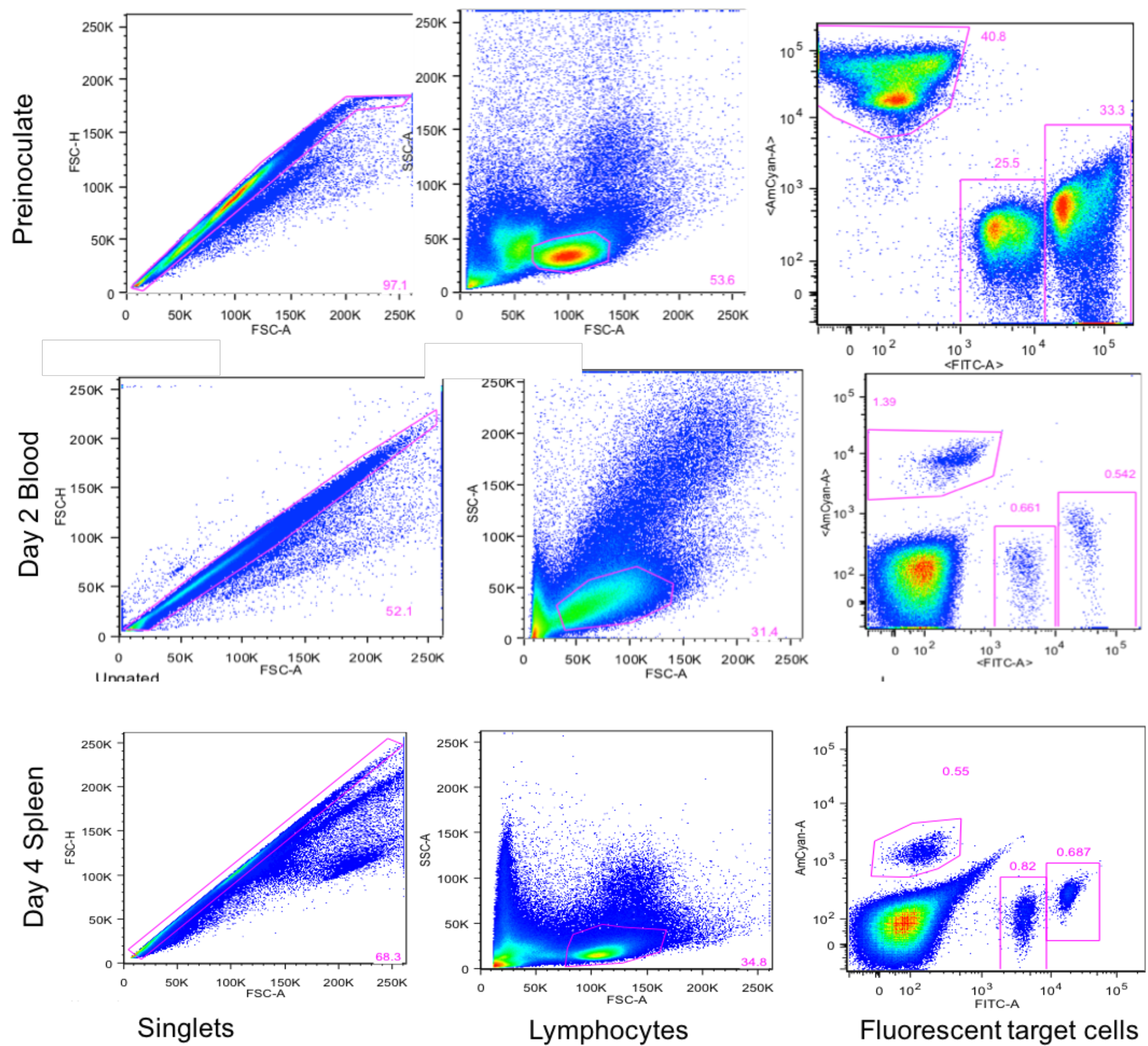


Figure 11. Monitoring survival of three different target cells inoculated in the same mouse. Spleen cells were labelled with two different concentrations of CFSE and Violet dye respectively, mixed 1:1:1 and inoculated i.v. into NK depleted mice to test resolution of the three populations at different time points. The labelled cell mixture was analysed at the time of inoculation, in the blood of inoculated mice on day two and in the spleen on day four after inoculation.

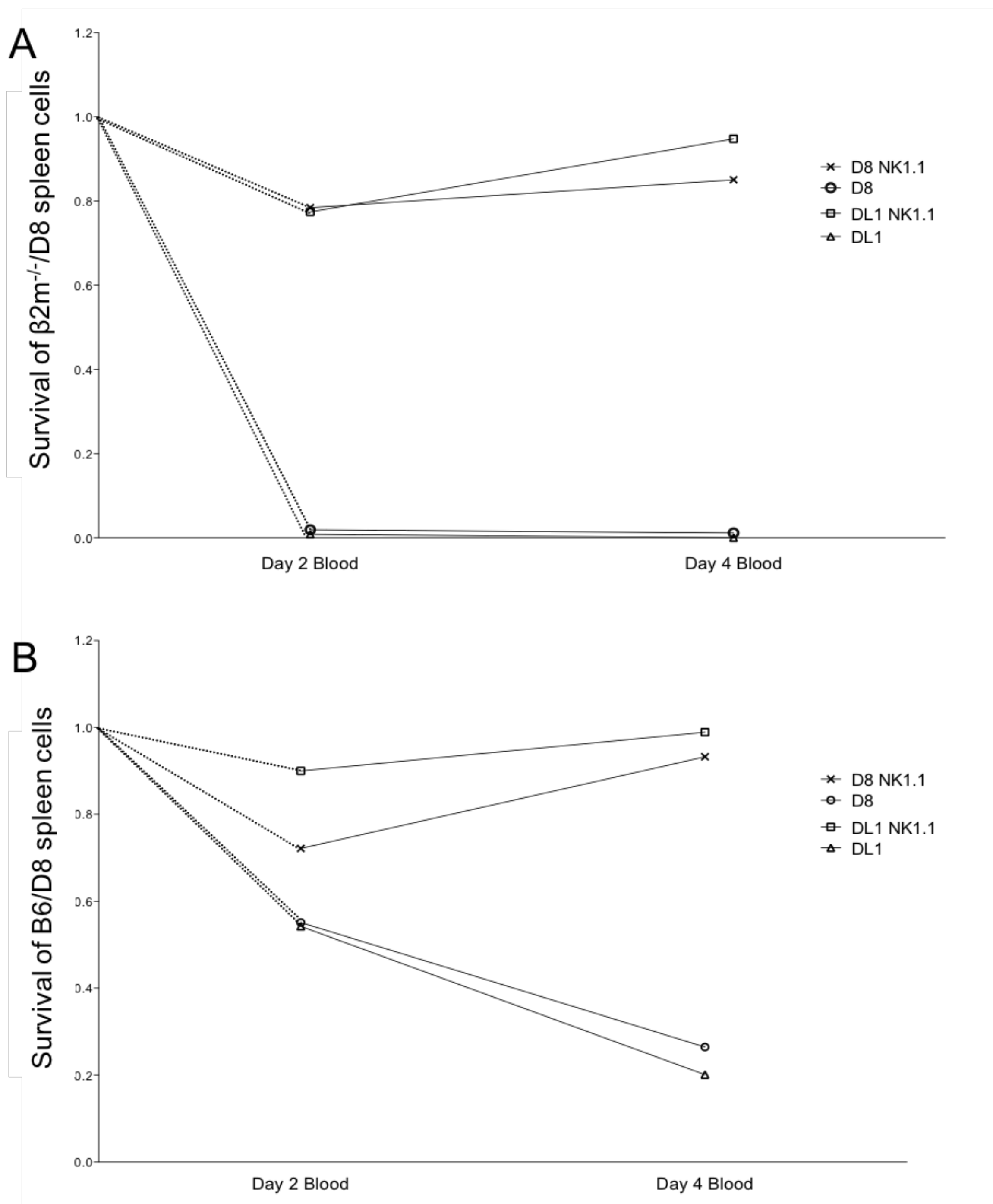
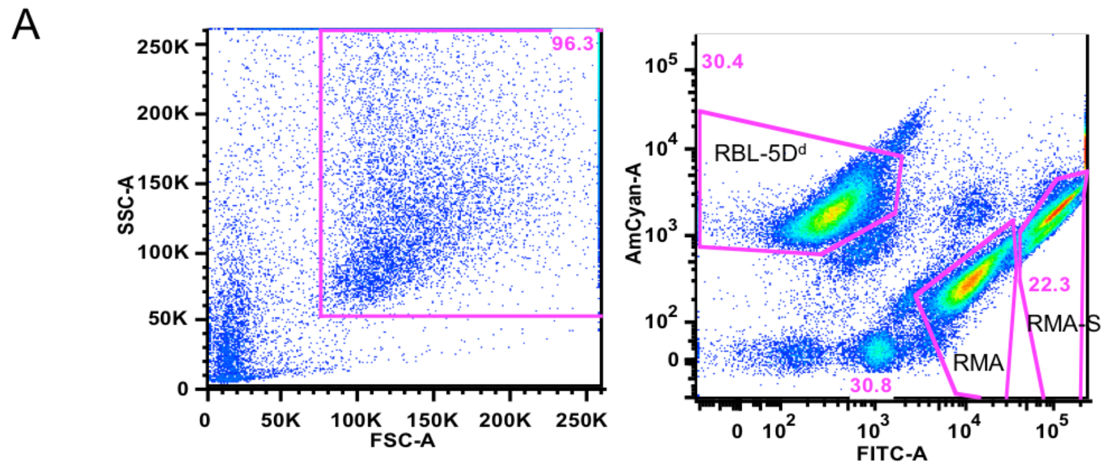
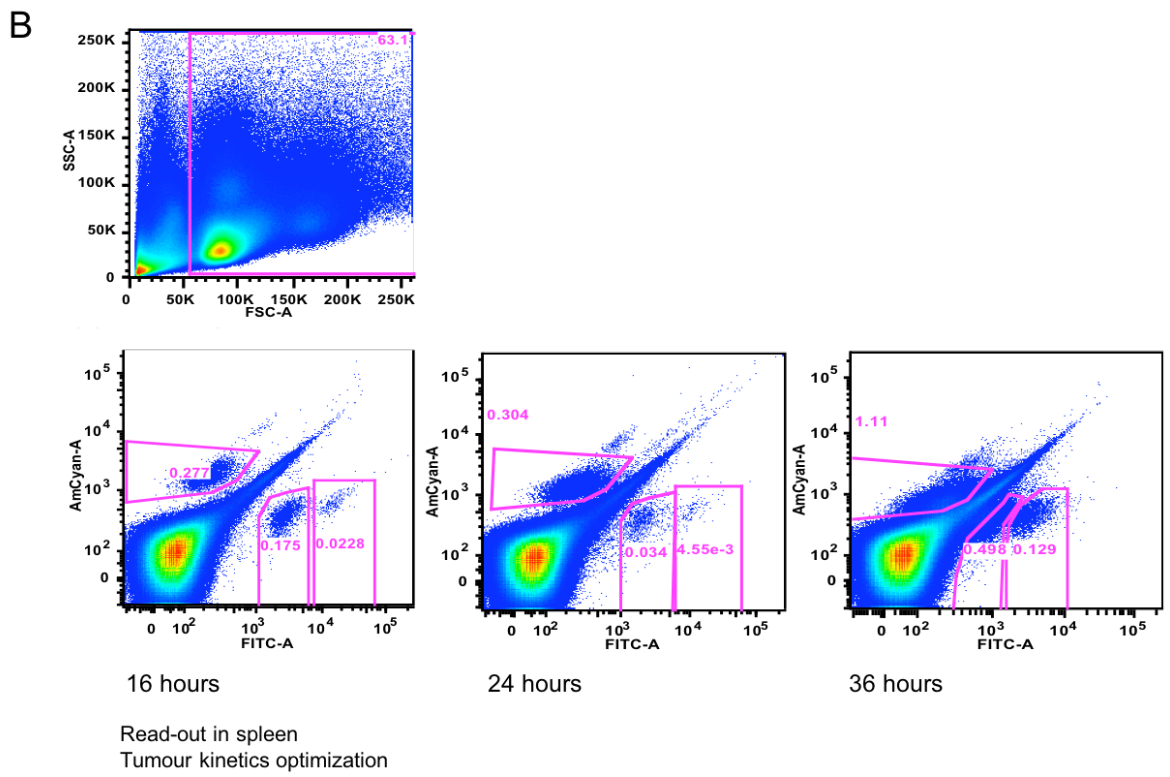


Figure 12. Kinetic analysis, missing self rejection of spleen target cells. Spleen cells from D8, B6 and $\beta2m^{-/-}$ mice were labelled with two different concentrations of CFSE and Violet dye respectively, mixed 1:1:1 and inoculated i.v. into D8 and DL1 mice, untreated or NK cell depleted. The labelled cell mixture was analysed at the time of inoculation, in blood on day two and day four. Graphs show corrected cell ratios for $\beta2m^{-/-}$/D8 (A) and B6/D8 (B). One representative experiment with means of two mice per group.





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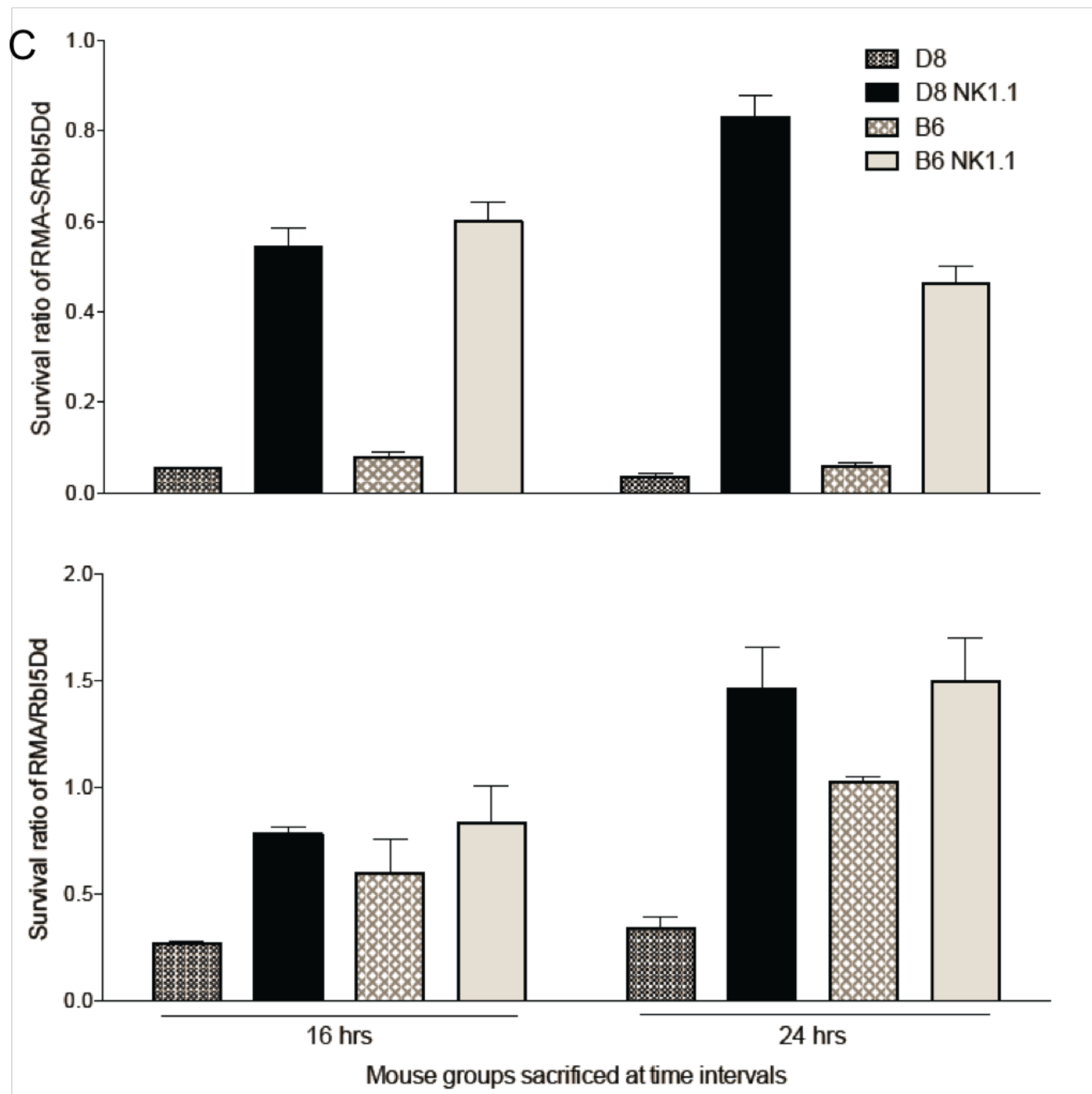


Figure 13. Kinetic analysis, missing self rejection of tumour target cells. Representative FACS plots showing: A. inoculate of a 1:1:1 mix of RBL-5Dd (Violet), RMA (CFSE-lo) and RMA-S (CFSE-hi), B. analysis in spleen of an inoculated B6 mouse at 16, 24 and 36 hours, C. Bar diagram showing the corrected ratio of target cells versus control cells: RMA-S/RBL5D^{d} (top) and RMA/RBL5D^{d} (bottom) at 16 and 24 hours. One experiment with two mice per group. Error bars, SD.

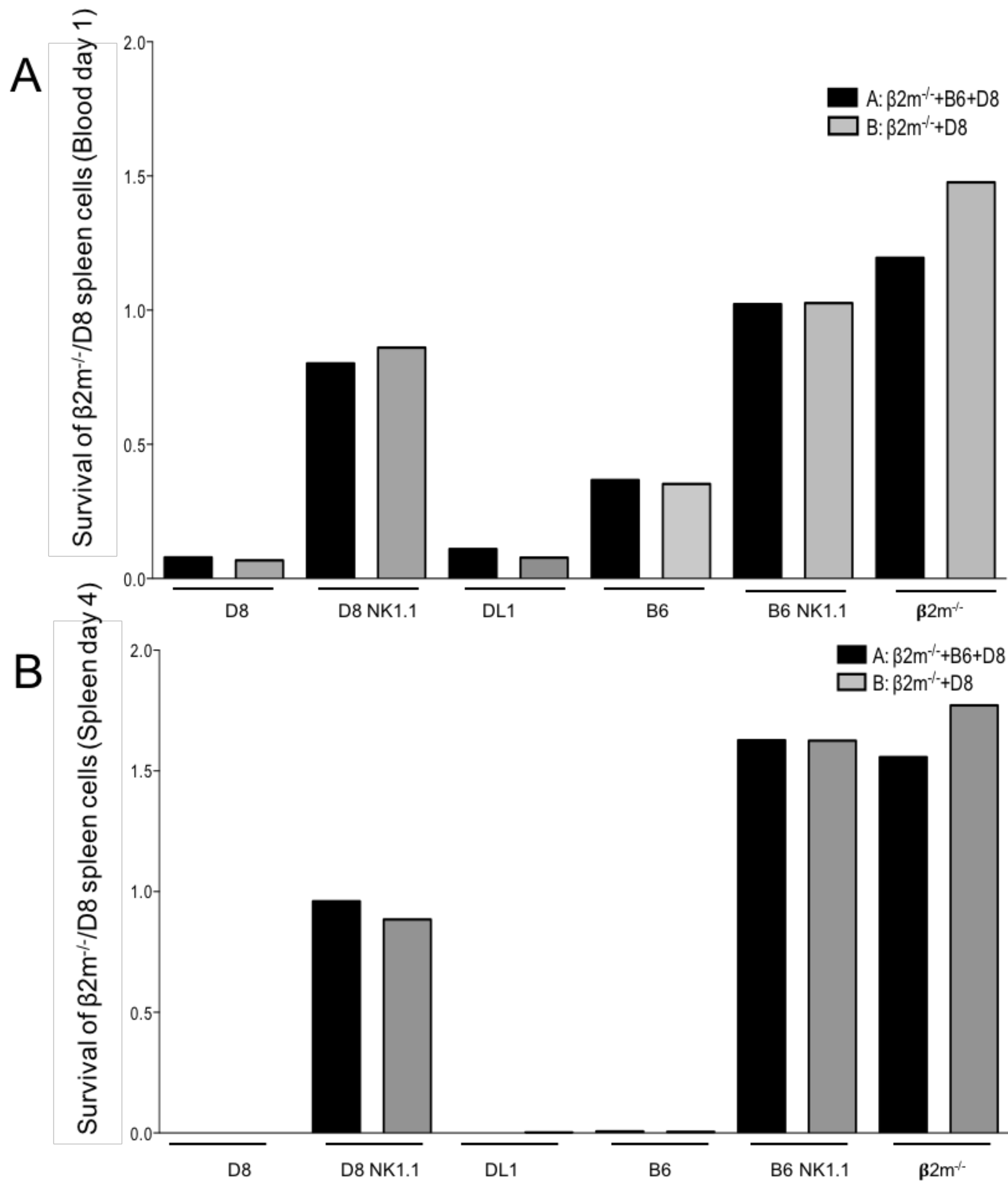


Figure 14. Comparison of two cell assay and three cell assay with spleen target cells. Spleen cells from β2m$^{-/-}$, B6 and D8 mice were labelled with two different concentrations of CFSE and Violet dye respectively, and either mixed β2m$^{-/-}$+B6+D8 (black bars) or β2m$^{-/-}$+D8 (grey bars) and inoculated i.v. into D8 and B6 mice, untreated or NK cell depleted, as well as DL1 and β2m$^{-/-}$ mice. The labelled cell mixture was analysed at the time of inoculation as well as in blood on day one (top) and spleen on day four (bottom). Graphs show corrected cell ratios for β2m$^{-/-}$/D8. One experiment with means of two mice per group.

4.3.3 Calculation of results

When injecting cells intravenously, there is sometimes variation between individual mice in the volume, i.e. the number of cells that get injected. The control cell population was used to correct for this. Thus, the final target-to-control ratios were corrected for the inoculated ratios to obtain the target cell survival ratio (for the formula, see materials and methods section). This method took into account internal controls for both inoculated and final readouts within

each mouse. It did not, however, account for any changes in the control population that might have occurred *in vivo* due to a number of reasons, e.g. rejection.

Another strategy for showing these data was also employed where survival of each cell type was measured as percent of total spleen cells, without relating it to any control population. This allowed for analysis without influence of a potential rejection of the control population. However, putative preferential homing of any cell type in a certain mouse strain, or variation in spleen size between individual mice may still confound this analysis.

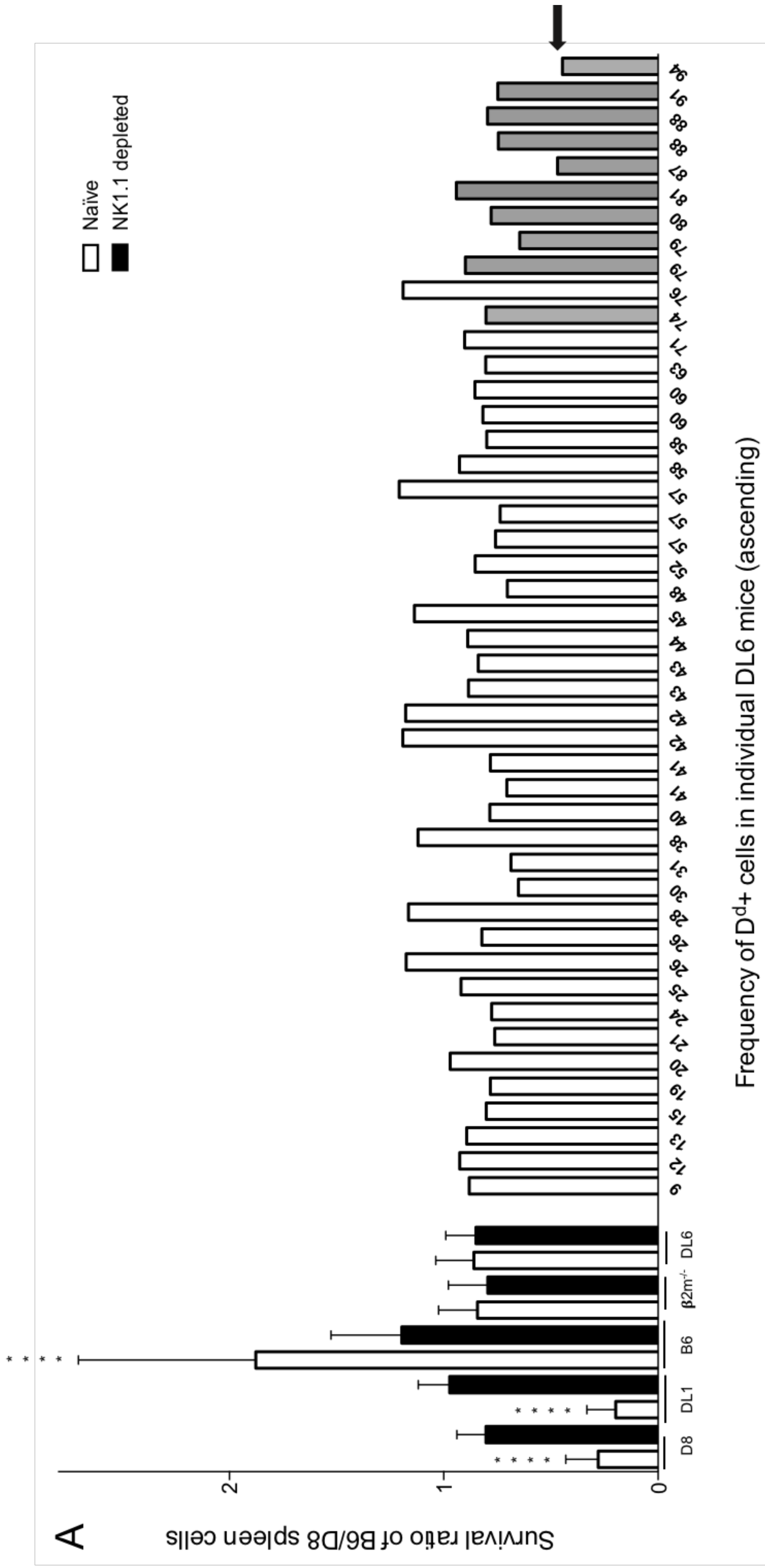
The data for spleen cell rejection is presented in both ways so that it can be analysed in different ways, accounting for different factors. Owing to the multiple variables involved, namely; various test-control cell combinations, several mouse strains with different MHC-I phenotypes, and possible involvement of other immune cell types especially in longer assays, this gives a fuller picture of the results.

4.4 NK CELLS IN DL6 MICE ARE TOLERANT TO CELLS LACKING D^d BUT EXPRESSING K^b AND D^b

With the set of typed mice and new or optimized assays described in the previous sections, we were in a position to test quantitative aspects of tolerance in the mosaic DL6 mice. It has been shown previously that such mice do not reject cells lacking D^d *in vivo* in assays measuring rejection of BM cells or outgrowth of subcutaneously inoculated tumour cells [92]. This indicated a complete NK cell tolerance to “allele specific missing self” in this setting of mixed MHC environment. However, neither of the assays used were quantitative, thus it was still possible that a low grade rejection of D^d negative cells would escape unnoticed. In addition, the study did not address “true tolerance” to resting healthy cells in untreated normal mice – the BM rejection assay involved rapidly proliferating haematopoietic cells grafted to sub-lethally irradiated mice. To study how the DL6 mice respond to healthy spleen cells having a partial lack in MHC-I molecules in a more quantitative assay, $K^{b+}D^{b+}D^{d-}$ and $K^{b+}D^{b+}D^{d+}$ (hereafter referred to as D^{d-} and D^{d+} cells respectively) spleen cells were labelled with different fluorescent dyes and DL6 mice were challenged with these targets. In these experiments, also differentially labelled cells, completely lacking MHC I, were included according to the method described above, in order to study general missing self responsiveness. The results for such cells are discussed below.

We reasoned along two hypotheses. 1) Mosaic mice would be completely tolerant towards D^{d-} target cells up to a certain frequency of host D^{d+} cells, when the few host D^{d-} cells were not sufficient to render the relevant NK subsets hyporesponsive. Mice with frequencies of D^{d+} cells above this threshold would efficiently eliminate D^{d-} spleen cells. 2) The tolerance would not be absolute in any of the mosaic mice, it would gradually change depending on the frequency of D^{d+} cells. This should be possible to read out as a correlation between the frequency of D^{d+} cells and a partial elimination of D^{d-} spleen cells.

The results did not fit any of these two hypotheses. Control mice D8 and DL1 ($K^{b+}D^{b+}D^{d+}$) rejected D^{d-} B6 spleen cells efficiently, displayed as a markedly reduced survival ratio. In contrast, there was no clear-cut rejection by any of the DL6 mice tested, irrespective of the frequency of D^{d+} cells (ranging from 9 to 94%; Figure 15A). There was no frequency threshold above which the pattern changed in a convincing way, although we note that the


A
Survival ratio of B6/D8 spleen cells
0
1
2
D8
DL1
B6
β2m-/-
DL6
Naïve
NK1.1 depleted
9 12 13 15 19 20 21 24 25 26 26 28 30 31 38 40 41 41 42 42 43 43 44 45 48 52 57 57 58 60 60 63 71 74 76 79 79 80 81 87 88 88 91 94
Frequency of Dd+ cells in individual DL6 mice (ascending)

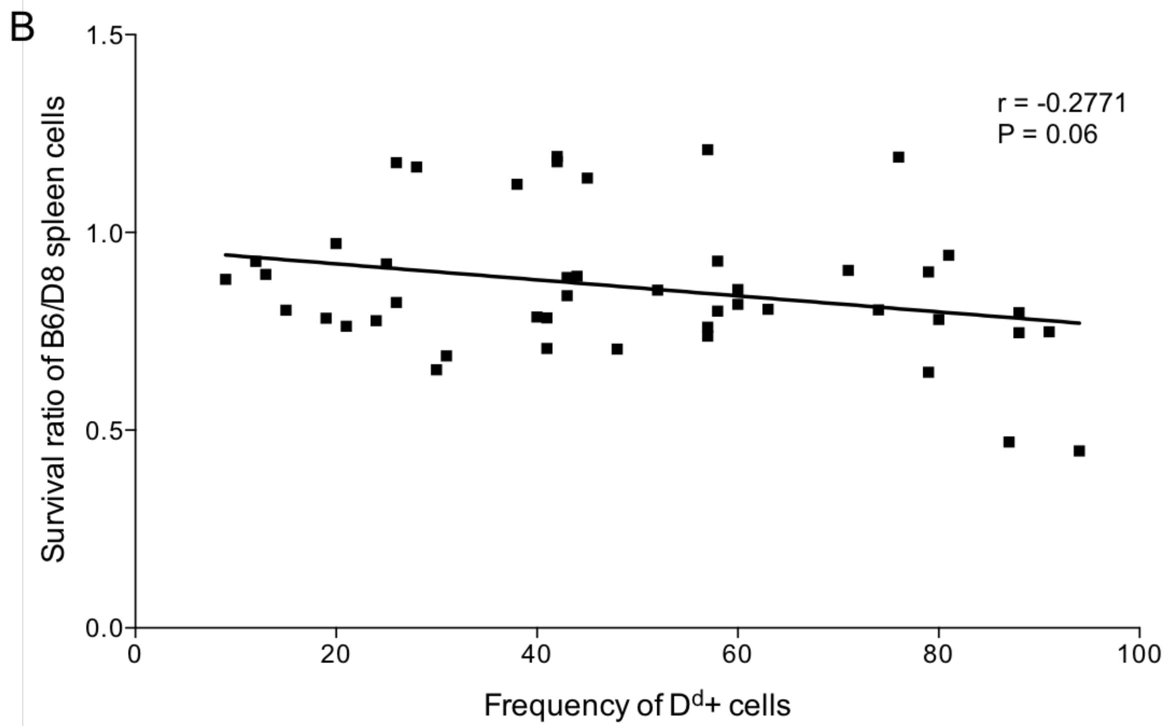


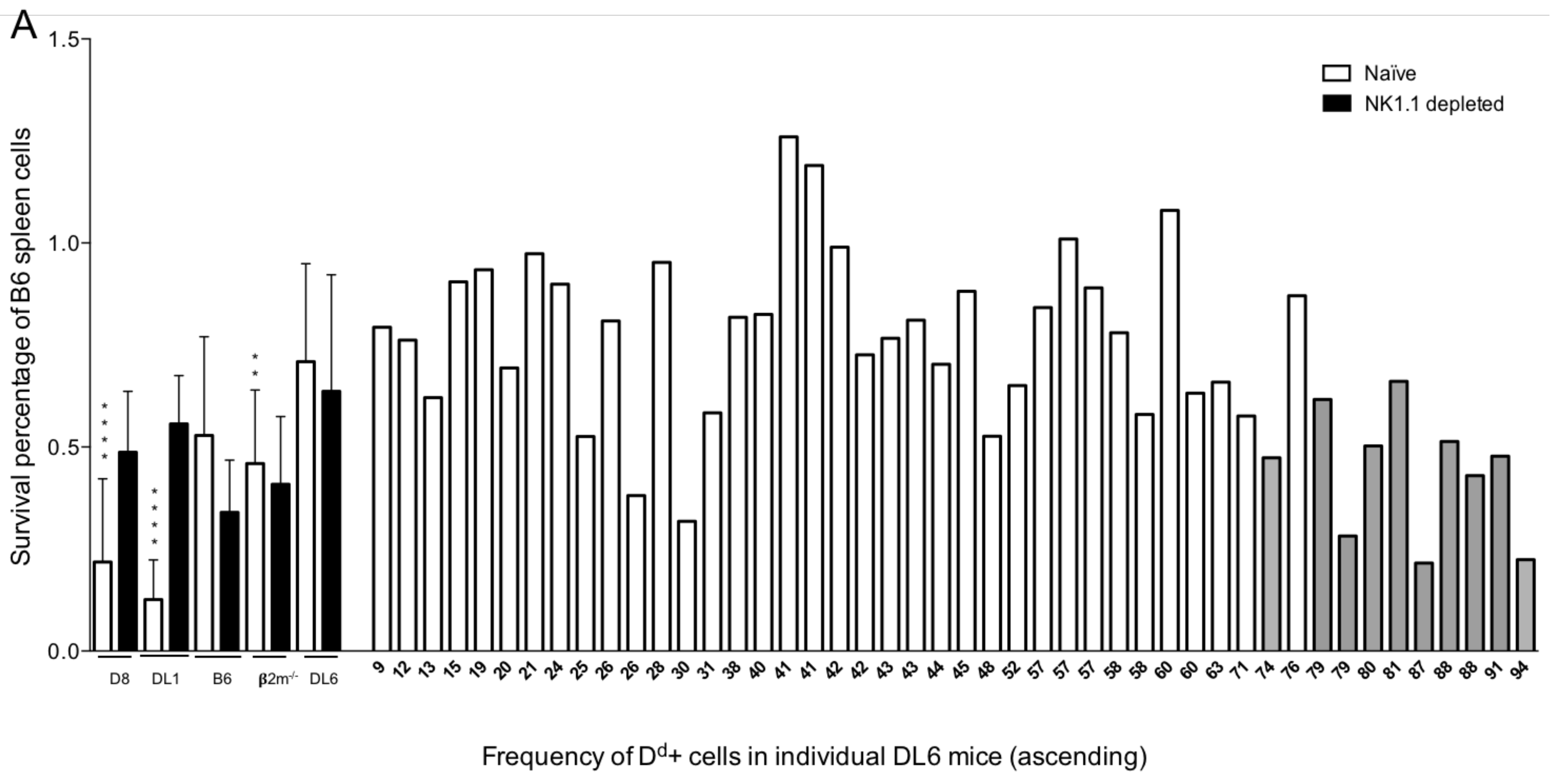
Figure 15. Relative survival of B6 vs. D8 spleen cells in DL6 mice with different frequencies of D^{d+} cells. Fluorescence labelled $\beta 2m^{-/-}$ (not shown), B6 (target) and D8 (control) cells were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as a corrected ratio of target cells versus control cells. Survival of target cells was analysed in spleens four days after inoculation. A. The left part of the figure shows means of mice within each group and the right part of the figure shows all individual DL6 mice from the DL6 group (shown as a mean value to the left) with frequency of D^{d+} cells denoted on the x axis. DL6 Ly5.1 mice are denoted by grey bars. The figure shows a compilation of 6 experiments with at least 8 mice in each group, except 6 mice in DL1, 4 in DL1 NK depleted, 46 in DL6 and 19 in DL6 NK depleted group. Error bars denote SD. The naïve DL6 group was statistically compared with all other naïve groups and DL6 NK depleted group. Statistically significant differences are denoted: ****, $P < 0.0001$. The black arrow indicates the rejection cut-off (0.469) calculated as mean+2SD of DL1 control. B. The figure shows B6/D8 ratio (y axis) in individual DL6 mice plotted against frequency of D^{d+} cells (x axis). The figure shows the same data for individual DL6 mice as in A.

only DL6 mouse which could be formally classified as a “rejector” (arbitrarily defined as a survival ratio of B6/D8 cells > mean + 2 SD of DL1 mice) also had the highest frequency of D^{d+} cells (94%; Figure 15A). Furthermore, there was no significant correlation between the frequency of D^{d+} cells and the survival of D^{d-} spleen cells in a linear regression analysis (Figure 15B). The rejection in D8 and DL1mice was NK cell dependent as mice depleted of NK cells did not reject such cells. We conclude that tolerance to D^{d-} normal cells is robust in mosaic mice, even when the frequency of D^{d+} cells is high. This indicates that NK cell education is induced or maintained through interactions with multiple cells, where even few cells deficient for an inhibitory MHC ligand will dominantly determine tolerance towards this phenotype.

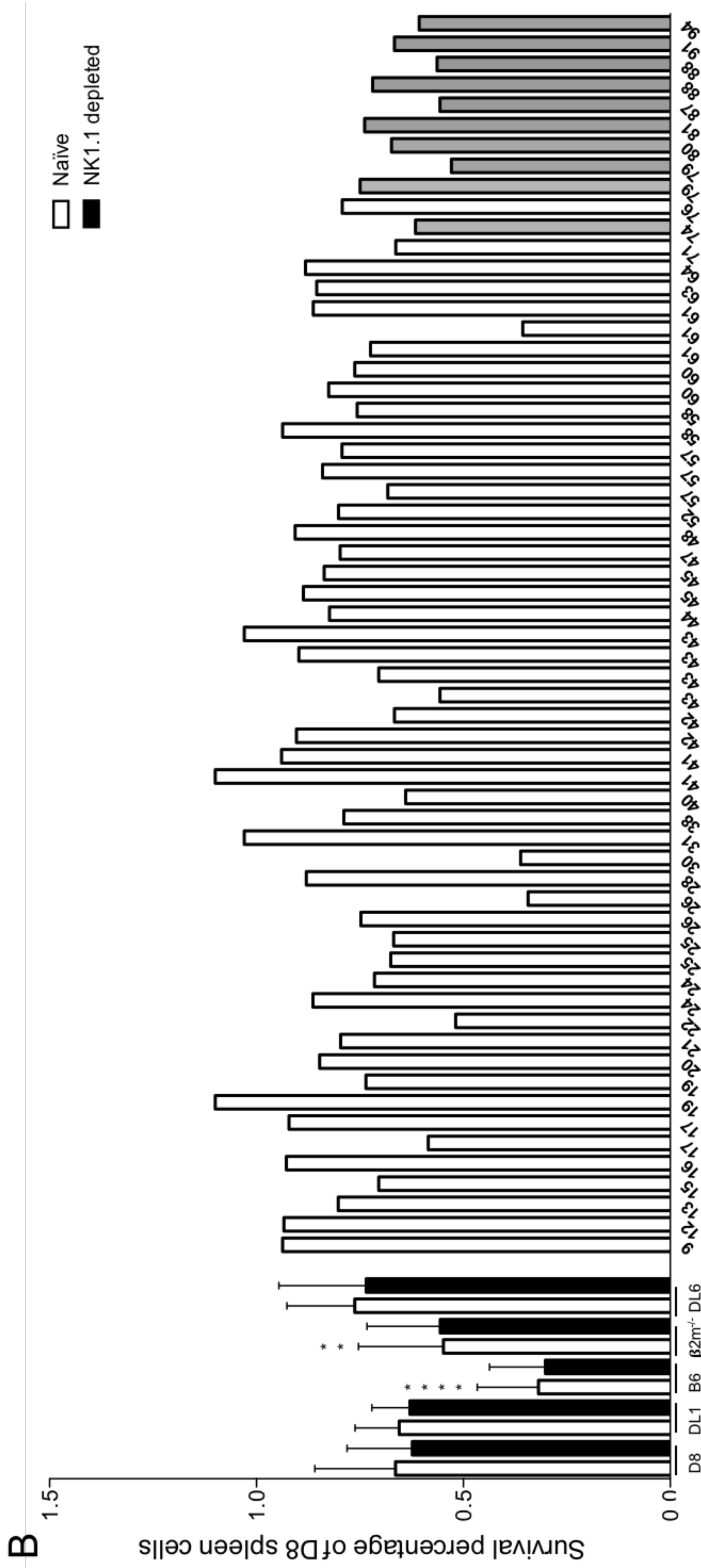
The pattern observed in one of the controls of these studies raised a concern about the use of relative survival ratios calculated against the D8 control target population. In B6 mice, there was a high survival ratio, frequently above 1, suggesting that the control population in the assay (i.e. D8 cells) had decreased. Such a decrease would lead to a survival ratio above 1 for the test population, in this case, the syngeneic B6 cells. This to some extent was observed for NK cell depleted B6 mice as well, pointing out that an NK cell dependent and/or independent rejection of the D8 target population (D^{d+}) may have occurred. It should be noted that $Ly49D^{+}$ NK cells in B6 mice are able to reject D^{d} expressing (D8) cells in bone marrow rejection assays in irradiated mice [233], also fully allogeneic BALB/c $H\text{-}2^{d}$ spleen cells have been shown to be rejected in an DAP12 dependent manner (indicating involvement of Ly49D) in B6 mice [117]. Other cytotoxic cells e.g. $CD8^{+}$ T cells in the second line of defence, have slower time kinetics, and these cells may play a role during longer assays.

To analyse if rejection of D8 cells could be the cause of the observed high survival ratio, not only in B6 mice but possibly also in DL6 mice (which could conceal a potential rejection of D^{d-} B6 cells), we also calculated the results as "percent remaining labelled B6 and D8 cells of the total spleen cells" rather than as a survival ratio (Figure 16A and 16B respectively; please see materials and methods as well as the section on refinement of *in vivo* rejection assay above for detailed presentation of these calculation methods). As expected, the percent remaining B6 cells was reduced in D8 and DL1 groups in an NK cell dependent manner, while B6 and DL6 mice did not show any such reduction (Figure 16A). In contrast, the survival of D8 spleen cells was clearly reduced in B6 mice with and without NK cell depletion, while the other mouse strains did not show any such reduction (Figure 16B). To test if $CD8^{+}$ T cells could play a role, we depleted such cells in a control experiment (Figure 17). There was a trend towards higher percent remaining D8 spleen cells and lower B6/D8 ratios in B6 mice after $CD8^{+}$ T cell as well as NK cell depletion, reflecting better survival of D^{d+} D8 cells in these control groups, even if no statistically significant differences were observed. For DL6 no similar trend could be observed. In conclusion, B6 mice seem to reject D8 cells in a not yet fully characterized manner, possibly several cell types are involved. Importantly, the results showed that in DL6 mice, there was no rejection of D8 spleen cells, thus these cells could be used as a control population to calculate ratios which reflect survival of the test populations.

Figure 16. Survival of B6 and D8 spleen cells in DL6 mice with different frequencies of D^{d+} cells. Fluorescence labelled $\beta 2m^{-/-}$ (not shown), B6 and D8 cells were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as surviving B6 target cells (A) or D8 target cells (B) in percentage of total spleen cells. Survival of target cells was analysed in spleens four days after inoculation. The left part of the figure shows means of mice within each group and the right part of the figure shows individual DL6 mice (from the DL6 group shown to the left) with frequency of D^{d+} cells denoted on the x axis. DL6 Ly5.1 mice are denoted by grey bars. The figure is based on the same data as figure 15 and shows 5 experiments with at least 8 mice in each group, except 6 mice in DL1, 4 in DL1 NK depleted, 46 in DL6 and 19 in DL6 NK depleted group. Error bars denote SD. The naïve DL6 group was compared with all other naïve groups and DL6 NK depleted group. Statistically significant differences are denoted: **, $P < 0.01$; ****, $P < 0.0001$.



A
Survival percentage of B6 spleen cells
0.0
0.5
1.0
1.5
Naïve
NK1.1 depleted
D8
DL1
B6
β2m-/-
DL6
9 12 13 15 19 20 21 24 25 26 26 28 30 31 38 40 41 41 42 42 43 43 44 45 48 52 57 57 57 58 58 60 60 63 71 74 76 79 79 80 81 87 88 88 91 94
Frequency of Dd+ cells in individual DL6 mice (ascending)



B
Survival percentage of D8 spleen cells
1.5
1.0
0.5
0
D8
DL1
B6
β2m-/-
DL6
Naïve
NK1.1 depleted
Frequency of Dd+ cells in individual DL6 mice (ascending)

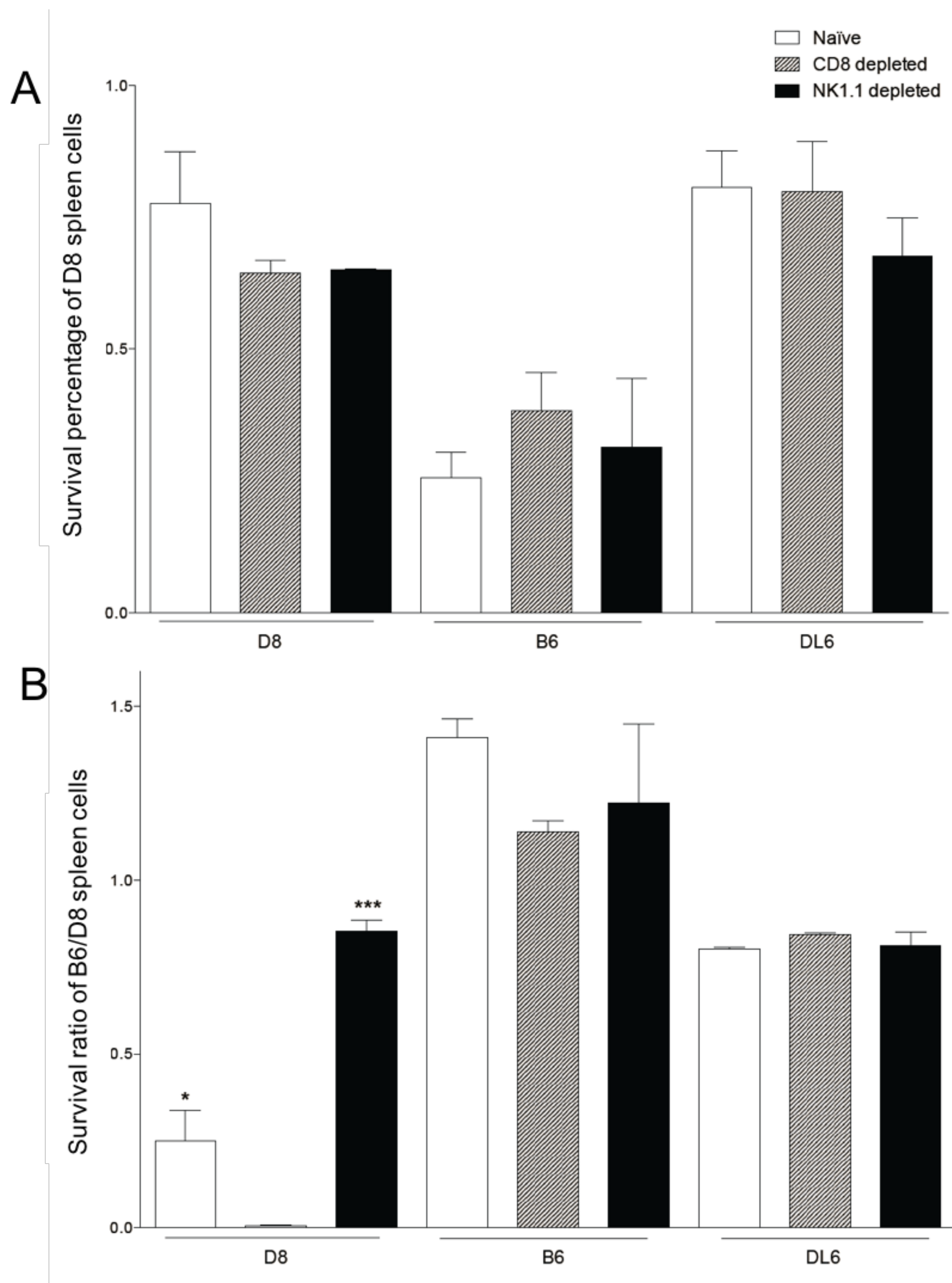


Figure 17. Analysis of the role of CD8^{+} *T cells and NK cells for survival of B6 and D8 spleen cells in a four day rejection assay.* Fluorescence labelled $\beta 2m^{-/-}$ (not shown), B6 and D8 cells were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-CD8 or anti-NK1.1 mAb. Results are displayed as surviving D8 target cells (% of total spleen cells) (A) or B6/D8 survival ratio (B). Survival of target cells was analysed in spleens four days after inoculation. The figure shows one experiment with means of two mice in each group. Error bars denote SD. The naïve group in each mouse strain was compared with its respective CD8 and NK1.1 depleted groups. Statistically significant differences are denoted: *, $P < 0.05$; ***, $P < 0.001$.

4.5 DL6 MICE SHOW A REDUCED REJECTION OF $B_2M^{-/-}$ SPLEEN CELLS COMPARED TO D8 AND DL1 MICE

The results thus indicated that all mosaic DL6 mice develop a complete tolerance to normal D^{d-} cells. We speculated that this might be due to hyporesponsiveness in one or more NK cell subsets (as defined by Ly49r expression) which would be highly responsive and “missing self reactive” in mice with normal D^d expression. We reasoned that such a hyporesponsiveness in several NK subsets due to the presence of a conflicting MHC-I environment (D^d mosaic) should lead to reduced overall rejection capacity towards MHC-I deficient cells. We further speculated that such a reduced rejection capacity might be quantitatively influenced by the frequency of D^{d-} cells in the mice, since this frequency would particularly influence the subset which would be missing self-reactive in mice with normal D^d expression. We thus set out to test whether DL6 mice would show reduced capacity for general missing self rejection, again with two possible models in mind. 1) This reduced capacity would only occur in mice with frequencies of D^{d+} cells below a certain threshold, 2) Increased number of D^{d+} cells would correlate with gradually increased general missing self responsiveness across the span of frequencies of D^{d+} vs. D^{d-} cells.

We could indeed observe a consistent, overall reduction in general missing self rejection capacity in DL6 mice (Figures 18, 19). This was most obvious in the early phase after inoculation of target cells, but the reduced rejection was not simply a matter of delayed kinetics; it persisted and was still present on day 4 (Figure 18). There was considerable variability, with a profound reduction in some mice, and less in other mice. However, rejection was never completely abolished, as shown by the comparison with the rejection pattern in $\beta_2m^{-/-}$ mice (Figure 19A). Although high and low responders could be found across the entire span of frequencies of D^{d+} cells in the DL6 mice, a fair (r = -0.57) and highly significant inverse correlation between this frequency and survival of the $\beta_2m^{-/-}$ target cells emerged in a linear regression analysis (Figure 19B). Furthermore, although we did not observe a clear-cut off threshold above which all DL6 mice responded as well as D8 and DL1 mice, we note that the majority (5/8) mice with 79% or more D^{d+} cells were within two standard deviations of the average $\beta_2m^{-/-}$ /D8 survival ratio observed for DL1 mice, our arbitrary cut-off for a normal, fully efficient general missing self rejection (Figure 19A). We further note that many but not all DL6 Ly5.1 mice were in this category of arbitrarily defined “complete rejectors”. Mice from this breeding were included in order to extend the number of mice with high frequencies of D^{d+} cells, but it cannot be formally excluded that the Ly5.1 mice differ in some other respect which influences the NK cell system.

Considering the correlation between frequencies of D^{d+} cells and the capacity for general missing self rejection of $\beta_2m^{-/-}$ cells, we also performed a linear regression analysis to explore any possible correlation between the survival of $\beta_2m^{-/-}$ and survival of B6 cells in the subset of experiments where both of these target cells had been included. As seen in Figure 20A, the data do not support such a correlation, despite the fact that the moderate inverse correlation (r value = -0.63) between frequency of D^{d+} cells and survival of $\beta_2m^{-/-}$ cells was seen also in this subset of experiments (Figure 20B), This is in line with the notion that tolerance to allele specific missing self (i.e. the D^{d-} phenotype of B6 cells) was complete regardless of the frequency of D^{d+} cells in the DL6 mice.

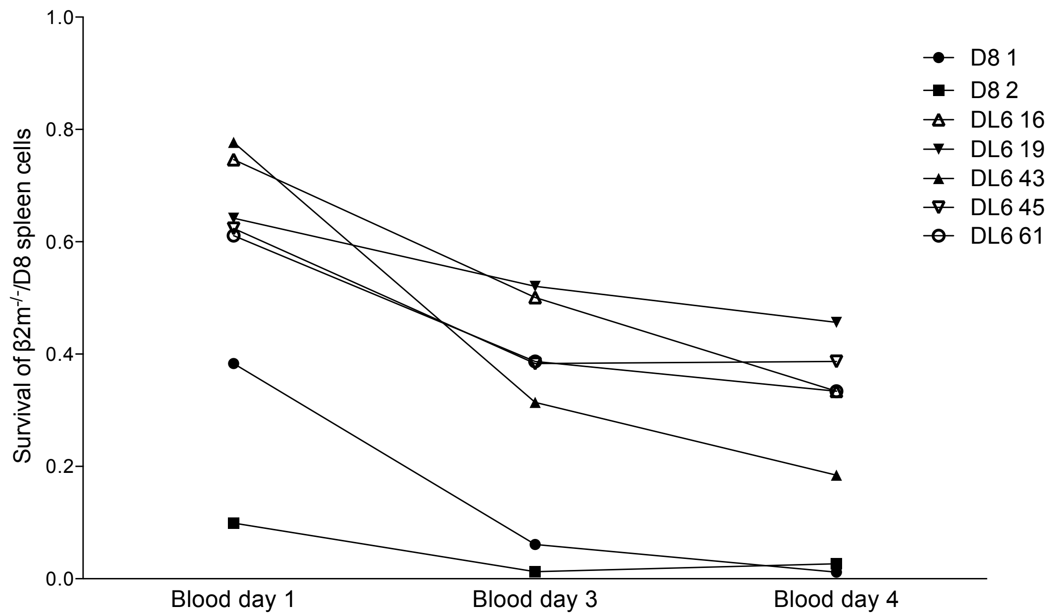
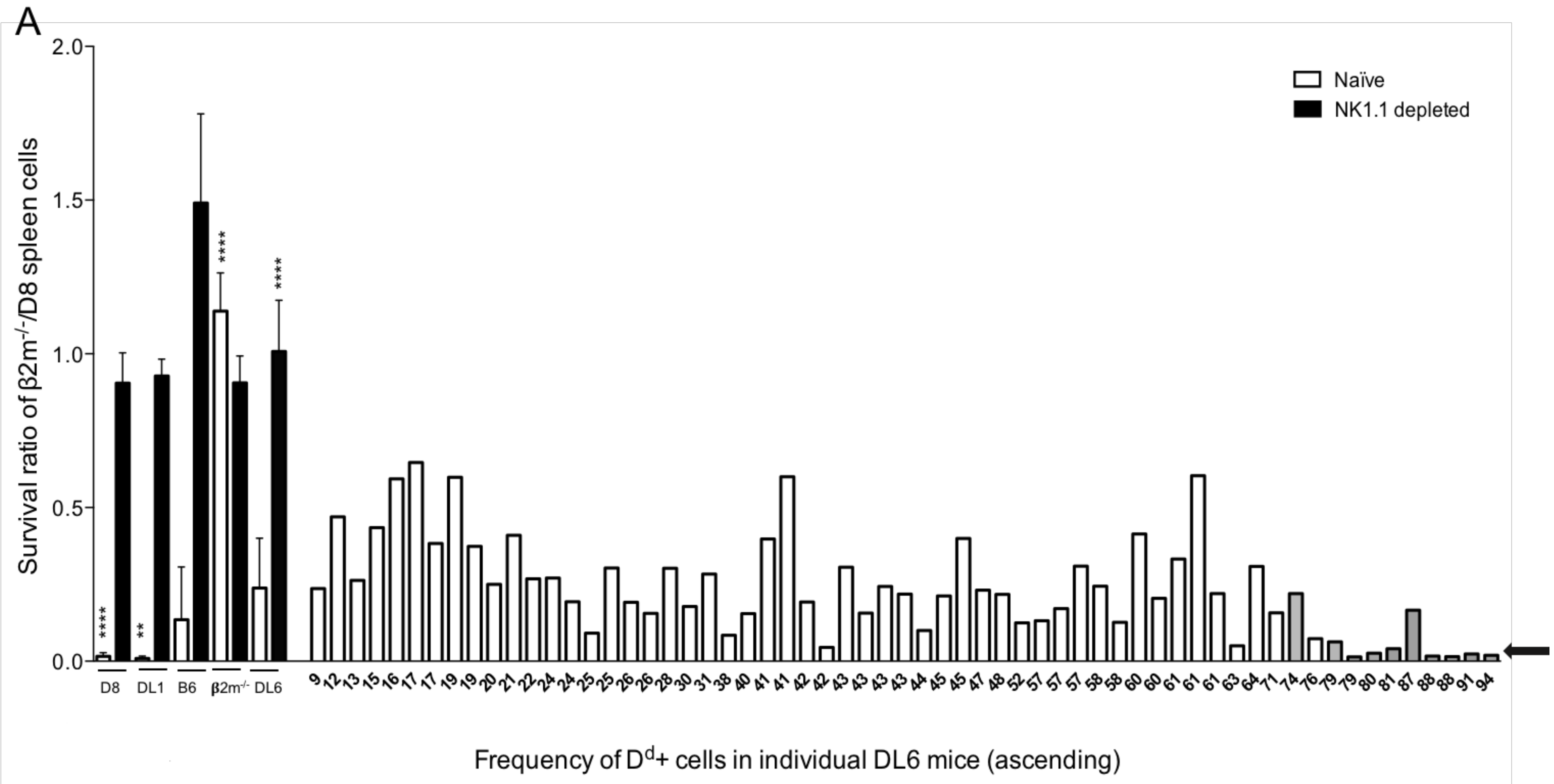


Figure 18. Kinetics of rejection of $\beta 2m^{-/-}$ spleen cells by D8 and DL6 mice. Fluorescence labelled $\beta 2m^{-/-}$ (target) and D8 (control) cells were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as a corrected ratio of target cells versus control cells. Survival of target cells was analysed in blood one to four days after inoculation. The figure shows two individual D8 control mice and five DL6 mice with frequency of D^{d+} cells denoted in the legend. The figure shows mice from one representative experiment out of 9.



A
Survival ratio of β2m-/-/D8 spleen cells
2.0
1.5
1.0
0.5
0.0
Naïve
NK1.1 depleted
****
**
****
****
D8
DL1
B6
β2m-/-
DL6
9 12 13 15 16 17 17 19 19 20 21 22 24 24 25 25 26 26 28 30 31 38 40 41 41 42 42 43 43 43 43 44 45 45 47 48 52 57 57 58 58 60 60 61 61 61 63 64 71 74 76 79 79 80 81 87 88 88 91 94
Frequency of Dd+ cells in individual DL6 mice (ascending)

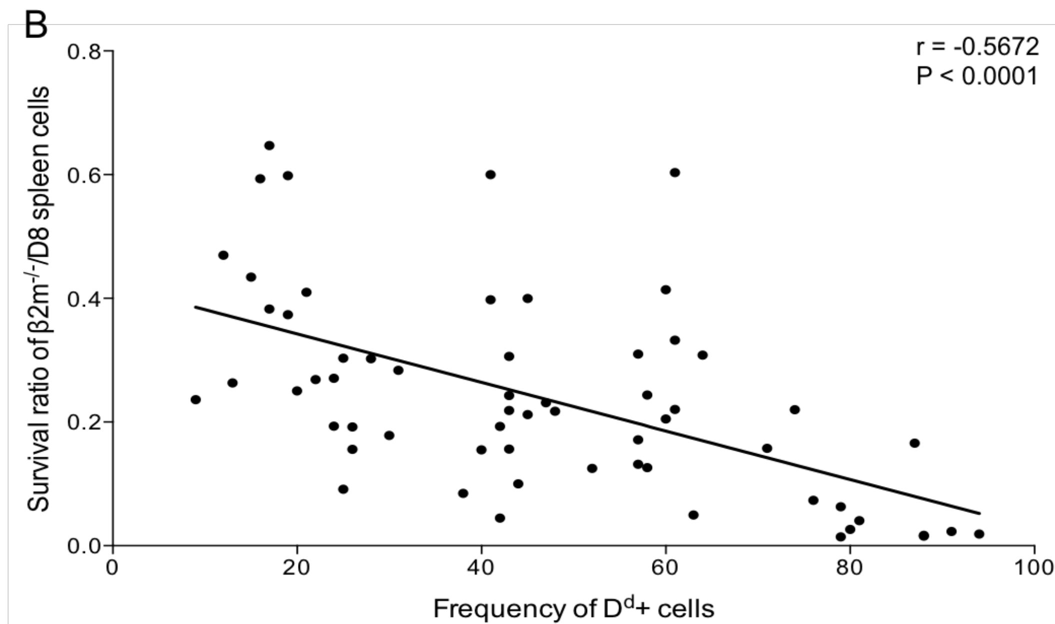


Figure 19. Relative survival of $\beta2m^{-/-}$ vs D8 spleen cells in DL6 mice with different frequencies of D^{d+} cells. Fluorescence labelled $\beta2m^{-/-}$ (test) and D8 (control) cells (in some cases also in mix with B6 cells; not shown) were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as a corrected ratio of target cells versus control cells. Survival of target cells was analysed in spleens four days after inoculation. A. The left part of the figure shows means of mice within each group and the right part of the figure shows all individual DL6 mice from the DL6 group (shown as a mean value to the left) with frequency of D^{d+} cells denoted on the x axis. DL6 Ly5.1 mice are denoted by grey bars. The figure shows 9 experiments with at least 8 mice in each group, except 6 mice in DL1, 4 in DL1 NK depleted, 61 in DL6 and 19 in DL6 NK depleted group. Error bars denote SD. The naïve DL6 group was compared with all other naïve groups and DL6 NK depleted group. Statistically significant differences are denoted: **, $P < 0.01$; ****, $P < 0.0001$. The black arrow indicates the rejection cut-off (0.025) calculated as mean+2SD of DL1 control. B. The figure shows $\beta2m^{-/-}$/D8 ratio (y axis) in individual DL6 mice plotted against frequency of D^{d+} cells (x axis). The figure is based on the same data for individual DL6 mice as in A.

We conclude that in an environment of mixed MHC phenotypes, the frequency of cells with a "complete" MHC-I expression (in this case $K^{b+}D^{b+}D^{d+}$) correlates with higher capacity for general missing self rejection by NK cells (as measured by rejection of cells with the $K^{b-}D^{b-}D^{d-}$ phenotype). Phrased differently, it thus appears that the presence of cells representing allele-specific missing self ($K^{b+}D^{b+}D^{d-}$) in the mixed MHC-I milieu, not only induces complete tolerance to allele specific missing self, but also "numbs" the overall responsiveness of the NK system as a whole. Overall, these findings are well in line with the predictions of the rheostat model for NK cell education [96] postulating that the MHC-I input received by inhibitory NK receptor gradually tunes the responsiveness of each NK cell. A more detailed discussion of this, as well as possible cellular mechanisms for this pattern will be presented below, taking into account also the results obtained from studies of tumour cell rejection.

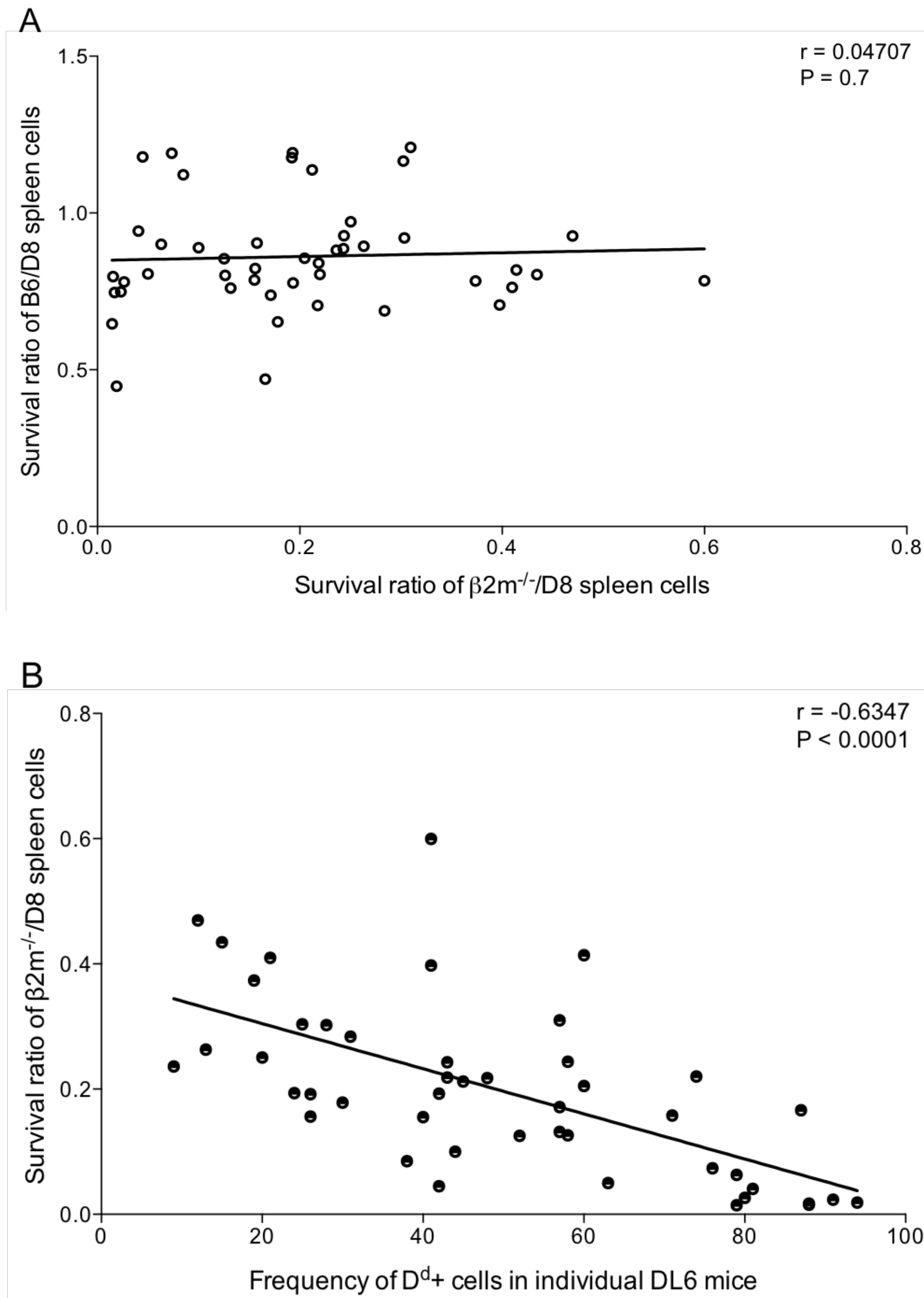


Figure 20. Survival of β2m$^{-/-}$ spleen cells correlated to survival of B6 spleen cells (A) or frequency of D^{d+} cells (B) in DL6 mice. Fluorescence labelled β2m$^{-/-}$ (test), B6 (test) and D8 (control) cells were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as a corrected ratio of target cells versus control cells. Survival of target cells was analysed in spleens four days after inoculation. A. The figure shows B6/D8 ratio (y axis) in individual DL6 mice plotted against β_2m$^{-/-}$/D8 ratio (x axis). B. The figure shows β2m$^{-/-}$/D8 ratio (y axis) in individual DL6 mice plotted against frequency of D^{d+} cells (x axis). The graphs are based on 46 DL6 mice, which represent a subset of the data in figure 19.

4.6 IMPACT OF MOSAIC MHC-I EXPRESSION IN THE NON-HAEMATOPOIETIC ENVIRONMENT, ON REJECTION OF SPLEEN CELLS REPRESENTING MISSING SELF

So far, the results indicated that NK cells developed robust tolerance to D^{d-} cells even if there were less than 15% of such cells in the host. This led to the question whether tolerance could develop even in mice where all cells in the haematopoietic system expressed D^d and where the mosaic expression of this molecule was restricted to the non-haematopoietic tissues.

We tested this by transplanting D8 bone marrow to DL6 mice with various proportions of D^d expressing cells, and then testing for rejection via missing self recognition. These D8 → DL6 chimaeras were thus tested for rejection of MHC-I sufficient B6 spleen cells lacking expression of D^d specifically, as well as MHC-I deficient $\beta_2m^{-/-}$ spleen cells. While the D8 → D8 chimaeras rejected B6 cells, the D8 → DL6 chimaeras showed strict tolerance, irrespective of the frequencies of D^d expressing cells in the recipient DL6 mice (Figure 21A). The same pattern was seen in the D8 → B6 chimaeras, where the recipients did not have a mosaic environment or any D^d expression at all. In contrast, there was an efficient rejection of the $\beta_2m^{-/-}$ spleen cells by the D8 → DL6 chimaeras, comparable to that seen in D8 → D8 chimaeras (Figure 21B).

From this experiment, we conclude that it is sufficient to have a fraction of non-haematopoietic cells lacking a specific MHC-I molecule, to induce tolerance for NK cell mediated allele specific missing-self responsiveness, even if all the cells in the haematopoietic system express this MHC-I molecule. However, the general responsiveness to cells completely lacking MHC-I molecules was not significantly reduced in this situation, suggesting differences in the regulation of rejection triggered by allele specific missing self versus the one triggered by complete missing self. It should be noted that this observation differs from what was observed in DL6 mice above (Figure 19A), where all mice showed a generally weakened (although not totally impaired) general rejection capacity towards MHC-I negative normal cells. One possible explanation is that the capacity to develop full responsiveness for general missing self rejection is more influenced by educating cells in the haematopoietic compartment, which represented a mixed D^{d+}/D^{d-} environment in the DL6, but not in the D8 → DL6 chimaeras. It should be noted that the difference may be quantitative rather than qualitative. The D^{d-} cells in the nonhaematopoietic environment may be sufficient to educate for impaired responsiveness to the extent that the critical NK subsets do not reject D^{d-} spleen cells, although in the absence of D^{d-} cells in the haematopoietic system, this impairment is not sufficiently profound to allow detection of generally reduced responsiveness against MHC-I deficient cells.

Overall, these results place emphasis on the D^{d-} cells in DL6 mice, once more, as the cells tutoring the lessons in education, but in this case focusing on non-haematopoietic cells. They also add to the notion of a very robust process for tolerance towards normal cells representing allele specific missing self.

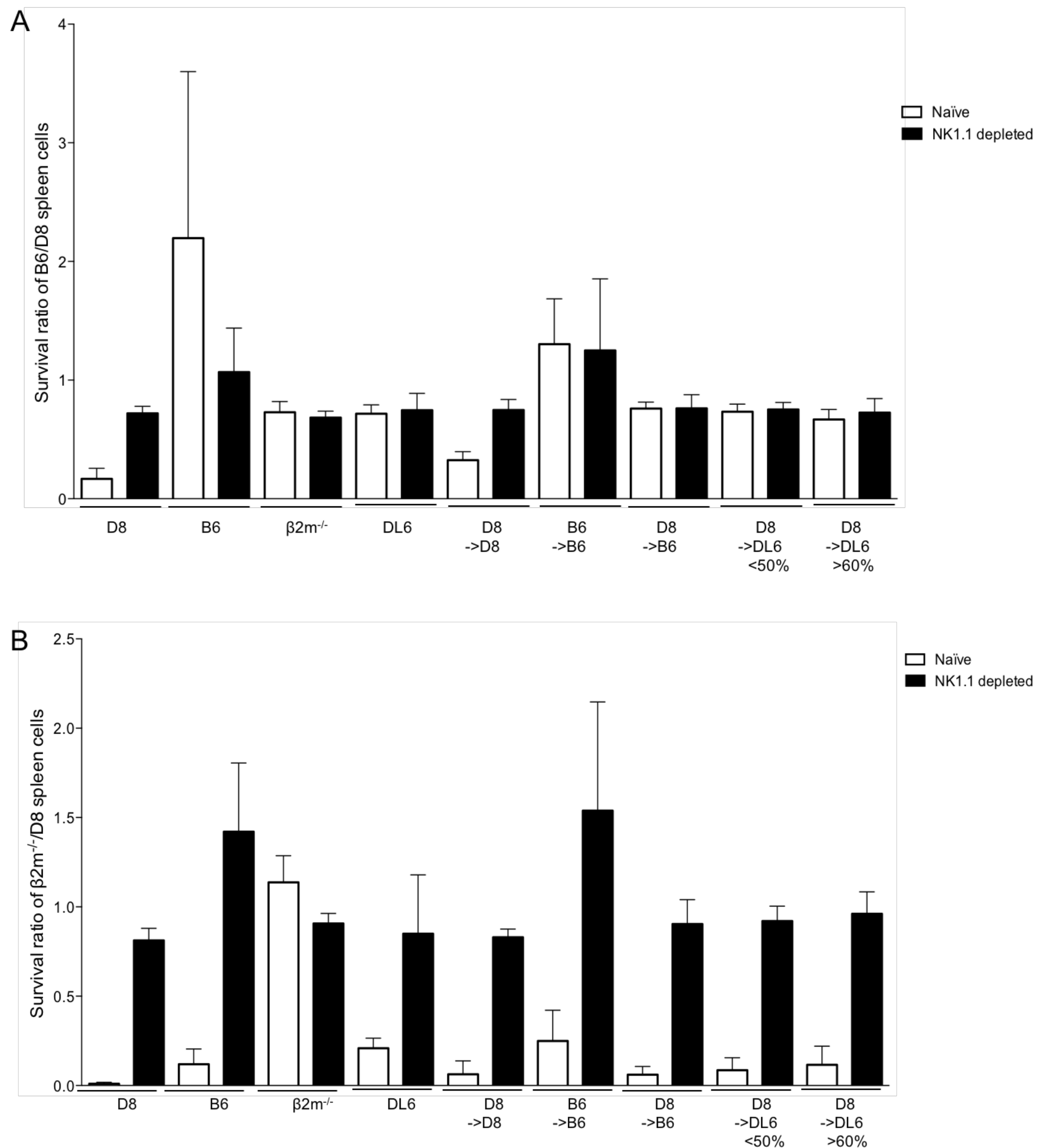


Figure 21. Analysis of $\beta 2m^{-/-}$ and B6 spleen cell rejection in D8 $\rightarrow$ DL6 bone marrow chimaeras. A mix of fluorescence labelled $\beta 2m^{-/-}$ (test), B6 (test) and D8 (control) cells was inoculated i.v. into bone marrow chimaeras and naïve controls that were untreated or treated with depleting anti-NK1.1 mAb. The D8 $\rightarrow$ DL6 chimaeras were divided in two groups, where the DL6 host carried <50% or >60% D^{d+} cells in peripheral blood before BM transplantation, as indicated on the x axis. Results are displayed as a corrected ratio of target cells versus control cells. Survival of target cells was analysed in spleens four days after inoculation. A. shows B6/D8 survival ratio and B. shows $\beta 2m^{-/-}$/D8 survival ratio. The figure shows 2 experiments with at least 4 mice in each control group and 12 mice in each chimaera group without NK1.1 depletion. Error bars denote SD. Statistical analysis was done in comparing A. D8$\rightarrow$B6 as well as the two D8$\rightarrow$DL6 chimaera groups with naïve DL6 mice and with their respective NK1.1 depleted control groups (n.s.), also no significant difference between D8$\rightarrow$DL6 chimaera groups; B. D8$\rightarrow$B6 as well as the two D8$\rightarrow$DL6 chimaera groups compared to D8$\rightarrow$D8 (n.s.), also no significant difference between D8$\rightarrow$DL6 chimaera groups.

4.7 MISSING SELF REJECTION OF TUMOUR CELLS IN DL6 MICE

We also studied tumour rejection in DL6 mice, with the methods developed and described above. Tumour cells often have more ligands for activating receptors on NK cells, resulting in higher susceptibility to killing and altered threshold for missing self rejection as already discussed above. We reasoned that this feature of tumour cells might result in a more sensitive system to detect quantitative alterations in tolerance induction in the DL6 mice. We thus decided to test the hypothesis that breaking of tolerance towards ''allele specific missing self'' would be revealed in DL6 mice with lower numbers of D^{d-} cells when tumour targets of the D^{d-} phenotype were used, even if the mice remained tolerant to normal cells of the D^{d-} phenotype as shown above. The three tumour lines used in this assay were the three genetic variants RBL5-D^d ($K^{b+}D^{b+}D^{d+}$, referred to as D^{d+}), RMA ($K^{b+}D^{b+}D^{d-}$, referred to as D^{d-}), and RMA-S with severely impaired MHC-I expression and perceived as MHC-I deficient by NK cells [78], referred to as MHC-I^-.

The tumour cell based assays showed rather large intra-experimental variations between individual mice, even in control groups with identical MHC-I expression, when calculated as percentage of surviving target cells per spleen cell. There could be several explanations for this, e.g. differential homing patterns of tumour as opposed to normal cells. Furthermore, there were no indications that B6 mice rejected the tumour cells used as internal controls: RBL-5D^d (data not shown). Since the important comparison is the one between the different tumour cells, the data are therefore shown only as survival ratios in relation to RBL-5D^d. This ratio showed only a small intra-experimental variability between individual mice when tumour cells were used. Again, we considered two possibilities: 1) Breaking of tolerance towards D^{d-} RMA cells above a certain frequency of D^{d+} cells in the DL6 mice 2) Increased number of D^{d+} cells would correlate with gradually increased allele specific missing self responsiveness against RMA cells, across the span of frequencies of D^{d+} vs D^{d-} cells.

The observed results were consistent with the latter model. While RMA cells were rejected in D8 and DL1 mice, there was considerable variability observed among DL6 mice, with no rejection in many mice and almost as efficient rejection as in D8 or DL1 by some mice (Figure 22A).

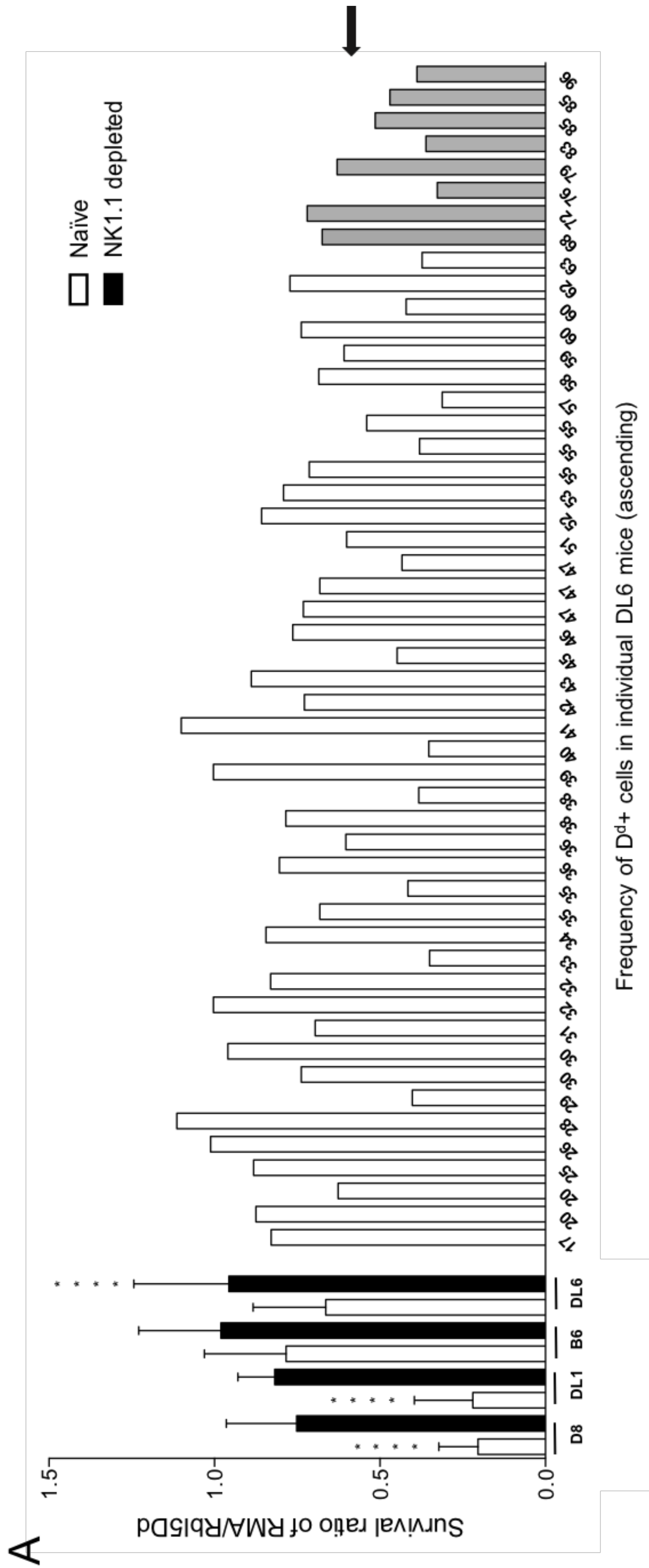
Although "high" and "low" responders could be found across the entire span of frequencies of D^{d+} cells in the DL6 mice, a weak but highly significant inverse correlation ($r = -0.46$) between this frequency and survival of the RMA target cells emerged in a linear regression analysis (Figure 22B). In some experiments, we analysed the survival in low responders after two days until the tumour cells were no longer detectable in spleen, and we could not observe any delayed rejection kicking in (data not shown).

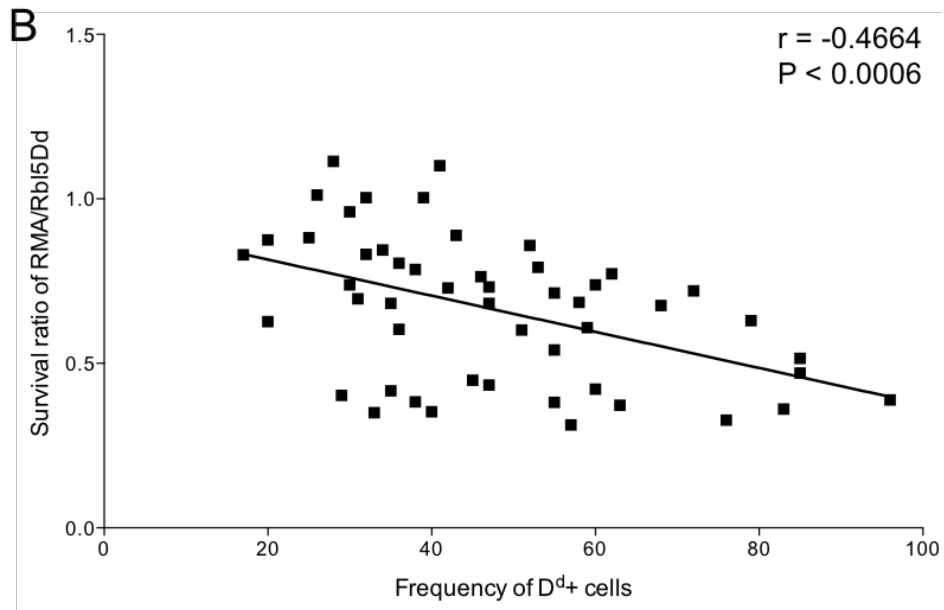
Using an arbitrary level of the average RMA/RBL-5D^d survival ratio + two standard deviations in positive DL1 control mice as cut-off for "rejection" ratio, we found "rejectors" across the span of frequencies of D^{d+} vs D^{d-} cells. However, all mice with ≥83% D^{d+} were qualified as "rejectors", suggesting that tolerance to D^{d-} tumour cells is broken at this threshold of just below 20% of D^{d-} cells. Again, we cautiously noted that all the mice with ≥83% D^{d+} cells were from the DL6 Ly5.1 breeding line.

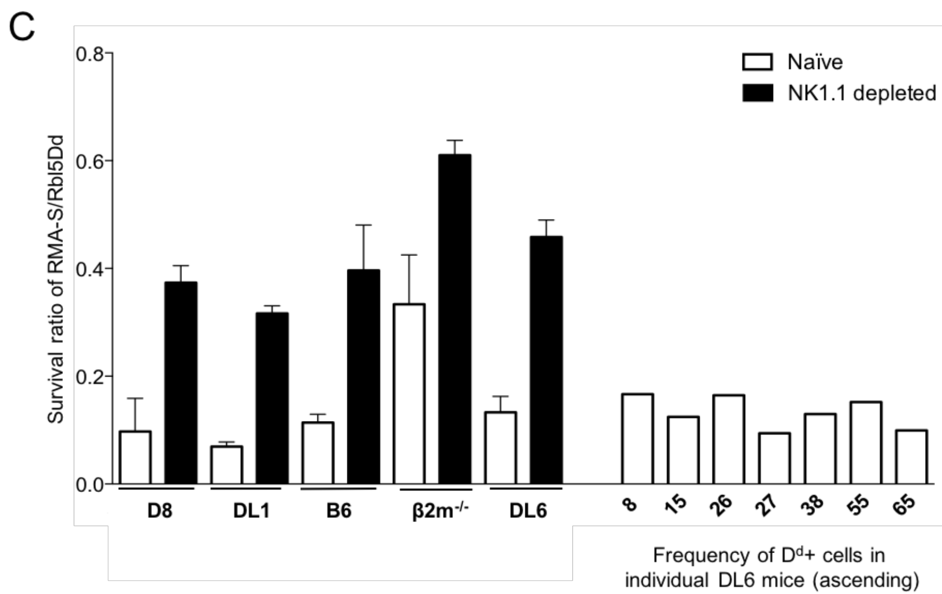
This led us to the expectation that DL6 mice would display a reduced capacity in general missing self reactivity also against MHC-I deficient tumour cells, corresponding to what we had seen with normal spleen cell targets. However, when we monitored the rejection of MHC-I deficient RMA-S tumour cells, DL6 mice rejected these cells very efficiently, comparable to D8 and DL1 mice. We studied this over a time line of 2 days and found that RMA-S rejection in DL6 mice began within 6-8 hours and was completed within 16- 24 hours (Figure 22C and D).

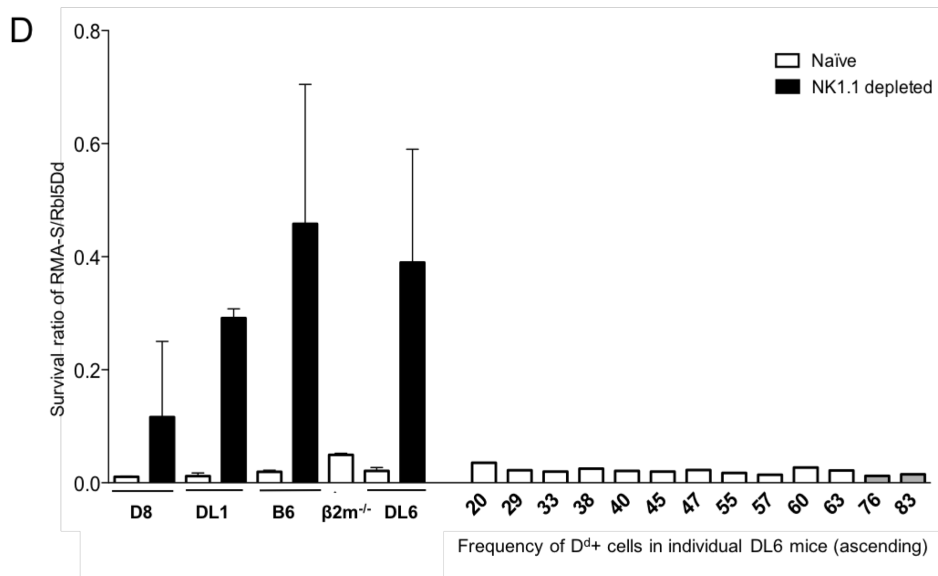
Interestingly, this pattern was different from the one we had observed with the healthy counterpart of RMA-S, i.e. MHC-I deficient spleen cells, which showed a clearly reduced missing self rejection in DL6 mice. This is reminiscent of what we observed in our studies of retuning by inhibitory receptor blockade as discussed above [222]. It thus appears that a mixed MHC-I expression in the host environment can differentially regulate reactivity to normal and tumour cells, similar to inhibitory receptor blockade or adoptive transfer of NK cells. To explain all of our observations in the DL6 mice, we propose that a higher expression of NK cell activating ligands on tumour cells brings them over a threshold where the general missing self hyporesponsiveness of DL6 NK cells cannot be detected in the assay for rejection of RMA-S tumour cells. This hyporesponsiveness is however sufficient to partly reduce the rejection capacity against MHC I deficient normal cells as well as RMA tumour cells (lacking one MHC I allele), and to completely abrogate rejection capacity against normal B6 spleen cells (lacking one MHC I allele).

Figure 22. Relative survival of RMA or RMA-S versus RBL5D^d tumour cells in short term rejection assays in DL6 mice. A-D. Fluorescence labelled RMA (test) and RBL5D^d (control) cells (A-B) or RMA-S (test) and RBL5D^d (control) cells (C-D) were inoculated i.v. into mice of different strains that were untreated or treated with depleting anti-NK1.1 mAb. Results are displayed as a corrected ratio of target cells versus control cells. A. Survival of RMA tumour cells in 18-24 hour assays. The figure shows a compilation of three experiments with a total of at least 6 mice in each control group, 51 DL6 mice and 21 DL6 NK depleted mice. The black arrow indicates the rejection cut-off (0.574) calculated as mean+2SD of DL1 control. B. The figure shows RMA/RBL-5D^d (y axis) in individual DL6 mice plotted against frequency of D^{d+} cells (x axis) and is based on the same data as in (A). C. Survival of RMA-S tumour cells in an 8 hour short term rejection assay. One experiment with 2 mice each control group, 7 DL6 and 4 DL6 NK depleted mice. D. Survival of RMA-S tumour cells in a 24 hour short term rejection assay. One experiment with 2 mice each control group, 13 DL6 and 7 DL6 NK depleted mice. A, C, D. The left part of the figure shows means of mice within each group and the right part of the figure shows individual DL6 mice (from the DL6 group shown to the left) with frequency of D^{d+} cells denoted on the x axis. DL6 Ly5.1 mice are denoted by grey bars. Error bars, SD. The naïve DL6 group was compared with all other naïve groups and DL6 NK depleted group. Statistically significant differences are denoted: ****, $P < 0.0001$.


A
Naïve
NK1.1 depleted
Survival ratio of RMA/Rbl5Dd
1.5
1.0
0.5
0.0
D8
DL1
B6
DL6
Frequency of D^{d}+ cells in individual DL6 mice (ascending)


B
r = -0.4664
P < 0.0006
Survival ratio of RMA/Rbl5Dd
Frequency of Dd+ cells


C
Naïve
NK1.1 depleted
Survival ratio of RMA-S/Rbl5Dd
D8
DL1
B6
β2m-/-
DL6
Frequency of Dd+ cells in individual DL6 mice (ascending)


D
Naïve
NK1.1 depleted
Survival ratio of RMA-S/Rbl5Dd
D8
DL1
B6
β2m-/-
DL6
Frequency of Dd+ cells in individual DL6 mice (ascending)

4.8 IMPACT OF MOSAIC MHC-I EXPRESSION IN THE NON-HAEMATOPOIETIC ENVIRONMENT ON REJECTION OF TUMOUR CELLS REPRESENTING MISSING SELF

The interpretation suggested in the end of the previous section led us to revisit the model where mosaic MHC-I expression was manifested exclusively in the non-haematopoietic compartment, i.e. the D8 → DL6 chimaera. As noted above, such chimaeras were completely tolerant towards the B6 spleen cells, representing allele specific missing self, although their general missing self responsiveness towards the $\beta_2m^{-/-}$ cells was fully efficient. If the same hierarchy applied in that situation as in the untreated DL6 mice, we would expect to detect rejection of RMA tumour cells (lacking only one allele of the cells in the haematopoietic compartment) in such mice, although it might be weakened compared to mice without mosaic MHC expression.

Our further experimental observations confirmed this (Figure 23). The D8 → D8 chimaeras efficiently rejected RMA cells. The two groups of D8 → DL6 chimaera showed significantly reduced rejection compared to these positive controls, however both of the groups (with < 50% and >60% D^{d+} cells in blood before BM transplantation respectively) showed significant rejection compared to B6 → B6 mice. One may also note that the D8 → DL6 mice with >60% D^{d+} cells rejected RMA cells more efficiently than the mice with <50% D^{d+} cells ($P < 0.05$). This suggests that the degree of mosaic expression in the non-haematopoietic tissues quantitatively influences the capacity for allele specific missing self rejection of tumour cells.

From this experiment, we conclude that it is not sufficient with a fraction of non-haematopoietic cells lacking a specific MHC-I molecule, to induce tolerance to NK cell mediated missing-self responsiveness against tumour cells lacking the same MHC-I molecule. This is in contrast to the responsiveness against normal cells representing allele specific missing self, where the corresponding situation led to complete tolerance, as discussed above (Figure 21A).

The situation with heterogenous MHC expression among non-haematopoietic cells exclusively is of course not directly relevant to any clinical situation arising in an immunotherapy protocol. However, the results are of importance from the perspective of basic understanding of NK cell education, as they support a general concept already emerging from the previous data presented in this thesis. The essential notion is that the outcome of interfering with the education process via perturbed sensing of MHC I varies depending on the cells used for the readout. Tumour cells can remain sensitive to the NK system under conditions where there is complete tolerance to normal cells with the corresponding missing self phenotype. There is at least one situation where this consequence of heterogenous MHC expression can be exploited in clinical immunotherapy: haematopoietic chimaerism occurring after bone marrow/stem cell transplantation or adoptive transfer. We therefore decided to set up an experimental system to assess the impact of mixed chimaerism in the haematopoietic compartment exclusively (with homogenous MHC expression in non-haematopoietic tissues) after bone marrow transplantation in mice.

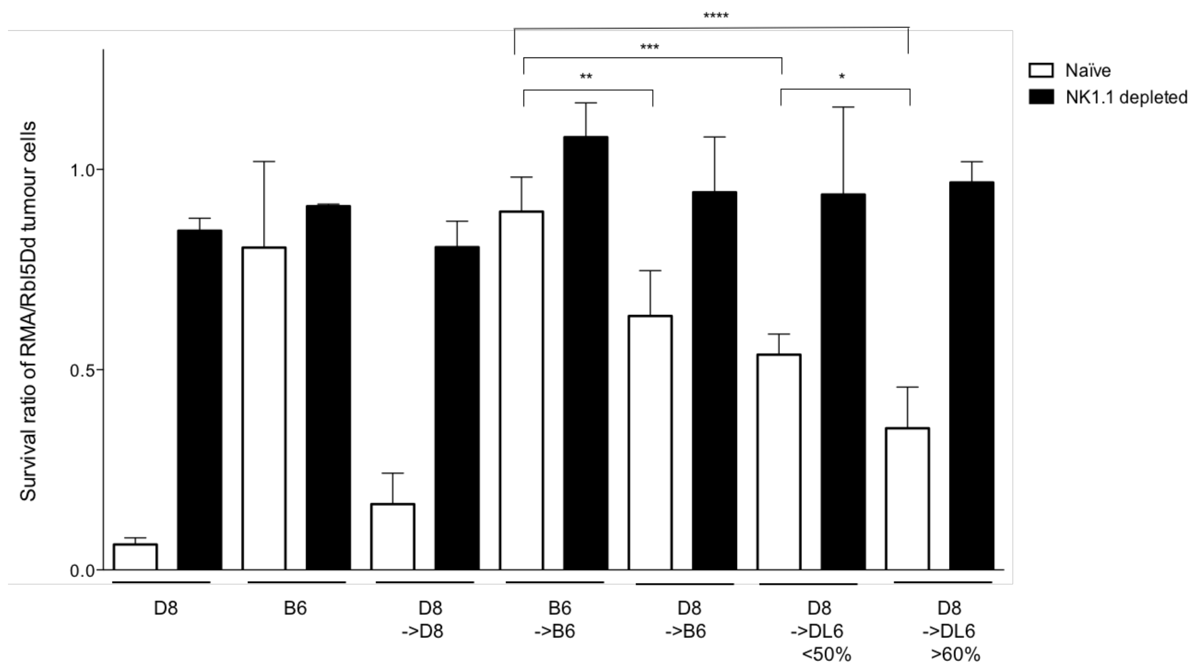


Figure 23. Analysis of RMA tumour cell rejection in D8 → DL6 bone marrow chimaeras. A mix of fluorescence labelled RMA (test) and RBL5-D^d (control) cells was inoculated i.v. into bone marrow chimaeras and naïve controls that were untreated or treated with depleting anti-NK1.1 mAb. The D8 → DL6 chimaeras were divided in two groups, where the DL6 host carried <50% or >60% D^{d+} cells in peripheral blood before BM transplantation, as indicated on the x axis. Results are displayed as a ratio of target cells versus control cells. Survival of target cells was analysed in spleens 20 hours after inoculation. The figure shows one experiment with 2 mice in each control group, and 4 mice in each chimaera group without NK1.1 depletion. Error bars denote SD. D8→B6 as well as the two D8→DL6 chimaera groups were compared to B6→B6 group, and D8→DL6 chimaera groups were compared with each other. Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

4.9 STUDIES ON TUMOUR CELL REJECTION IN MIXED BONE MARROW CHIMAERAS

The chimaera approach was also motivated from another point of view: the DL6 mice represented a limitation in the sense that the majority of them had a frequency of D^{d+} cells around 30-70%, while mice with extreme frequencies of D^{d+} cells were rare, even when Ly5.1 DL6 mice were used. In bone marrow chimaeras this should be possible to control, at least in theory. In order to study NK cell education in mice with very few D^{d-} cells we thus generated mixed bone marrow chimaeras where D^{d+} and D^{d-} bone marrow from D8 and B6 mice respectively was mixed in different predetermined percentages and transplanted into D8 hosts. This allowed us to assess not only the quantitative influence of the number of D^{d-} cells on missing self tolerance, but also to test the influence of D^{d-} cells when this phenotype was present exclusively in haematopoietic cells. All the D8 recipient mice in this chimaeric model expressed D^d in all of the non-haematopoietic cells.

When constructing these chimaeras, it was essential to prevent that D^{d+} NK cells (from the D^{d+} recipient mice as well from the D^{d+} donor mice) rejected haematopoietic cells from the

D^{d-} donor during reconstitution based on missing self. We tested several protocols for this NK cell depletion in pilot experiments. The bone marrow reconstitution was followed over time and the stability of the chimaeras was assessed by flow cytometry in blood cells after the initial lymphopenic period of about 10 days (Figures 24-26). We always observed an initial increase of the D^{d+} population compared to the administered ratio in the input mixture of marrows. However, after ten days, the chimaerism usually stabilized, at least in experiments where donor as well as recipients had been NK cell depleted (Figures 24 and 25). Interestingly, when donors but not recipients were NK cell depleted, we did not achieve stable chimaerism. The proportion of D^{d+} cells continued to increase throughout the life span of the mice (Figure 25). Altogether, the protocols allowed us to study chimaeras with a completely D^{d+} non-haematopoietic compartment and high frequencies of D^{d+} cells in the haematopoietic compartment, in a range extending beyond that offered by DL6 mice. In addition, we could compare the outcome of different experiments with 95% D^{d+}/ 5% D^{d-} mixed chimaerism, but where the mice appeared to be in a stable "tolerant" state in one case, and in an unstable "non-tolerant" state in the other.

When the stable mixed bone marrow chimaeras constructed with NK cell depletion of donor as well as recipient mice were challenged with the mixture of tumour cells, we observed a clear rejection of D^{d-} RMA cells representing allele specific missing self in the mice that had received 100% D^d expressing D8 marrow cells (Figures 24, 25). This rejection was markedly weakened already in mice containing as little as 4-8% of D^{d-} negative cells in the haematopoietic system after stabilization of chimaerism (Figures 24, 25). However, the rejection capacity was not completely abrogated, as noted when compared to B6→B6 chimaeras or normal B6 mice with no D^{d-} cells in the haematopoietic system. We also observed a significantly stronger rejection in chimaeras with 95% D^{d+} (range: 94-98%) cells, than chimaeras with 61% D^{d+} (range: 57-66%) cells in one experiment (Figure 25). The results are well in line with previous results where outgrowth of subcutaneous tumours was studied in the same type of bone marrow chimaeras [212]. In this study, the capacity to reject RMA tumours of mixed D8+B6 →D8 chimaeras containing less than 20% D^{d-} cells, was clearly reduced compared to D8→D8 chimaeras (tumour outgrowth in 17/24 and 0/13 mice, respectively). However, the former were still more resistant than B6 mice (outgrowth in 11/11 mice).

A different picture emerged in unstable chimaeras where only donors had been depleted of NK cells. All the mixed D8/B6 → D8 chimaeras (which had now developed to contain 94-95% D^{d+} cells, (range: 90-96%) showed strong rejection of RMA cells, comparable to that seen in D8 → D8 chimaeras (Figure 26).

From the mice developing unstable chimaerism, we conclude that the parallel reconstitution of D^{d+} and D^{d-} cells in the same host (D^{d+}) environment does not always lead to tolerance towards allele specific missing self. Similar results have been published with mixed B6 wild type and $\beta_2m^{-/-}$ bone marrow chimaeras. These mice gradually developed towards higher frequencies of B6 vs. $\beta_2m^{-/-}$ cells, a process which was accelerated if the NK cells were activated, e.g. by murine cytomegalovirus infection [39]. In the future, it would be of interest to explore exactly why the system sets off in this "non-tolerance" direction in certain situations, since this could be harnessed for anti-tumour therapeutic effects. Here, this lack of

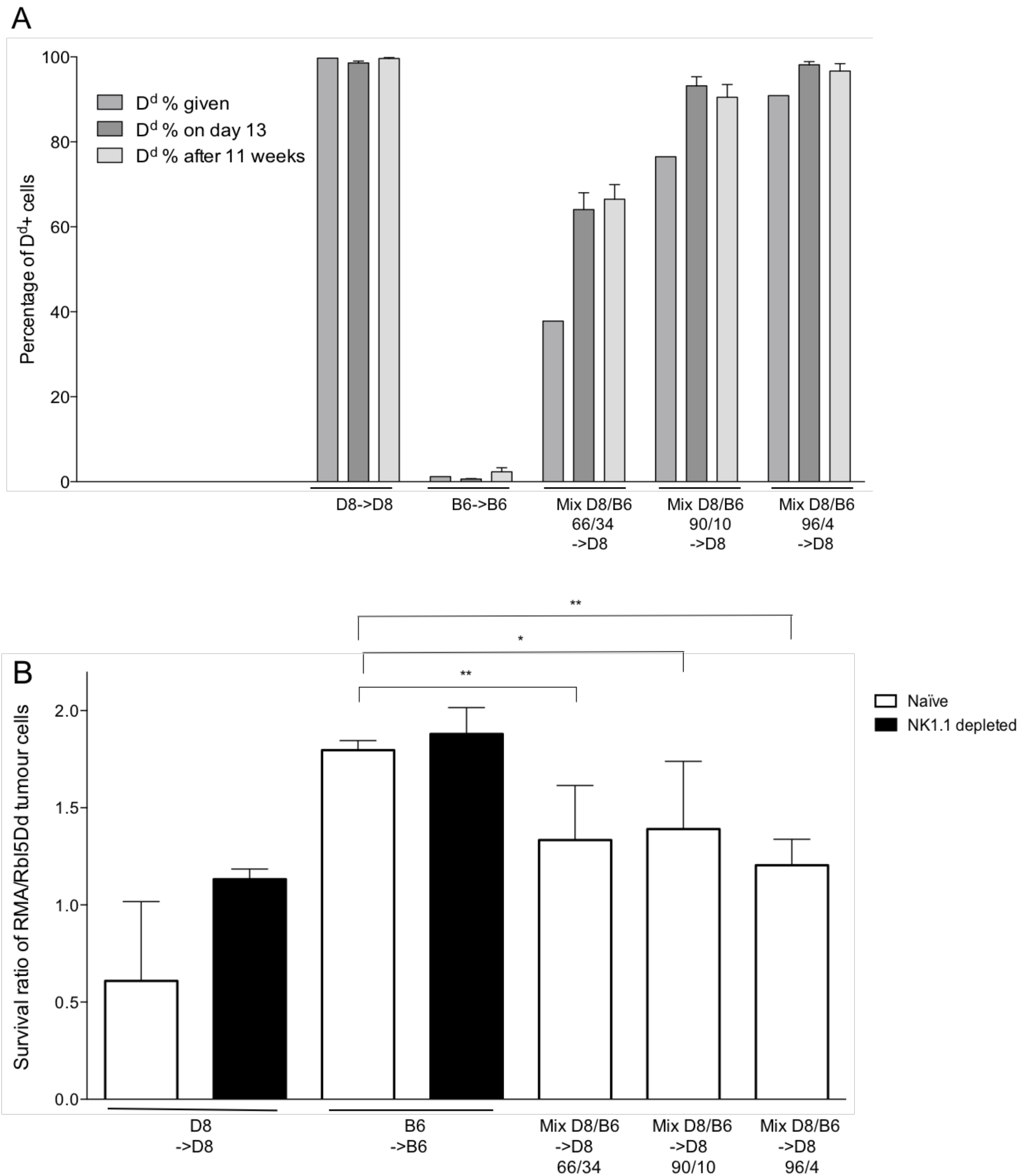
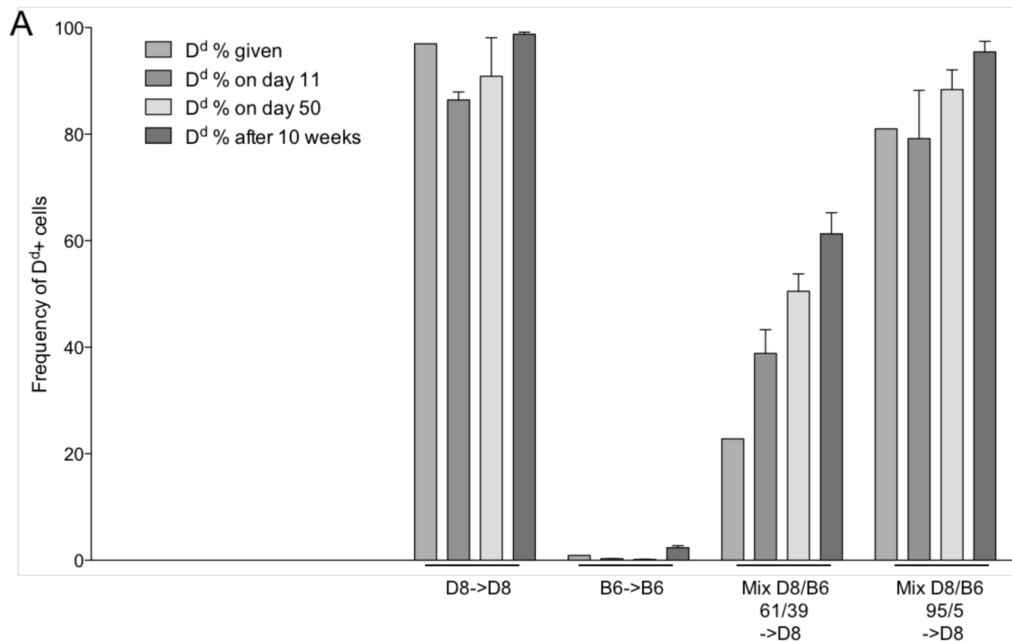


Figure 24. Analysis of RMA tumour cell rejection in stable mixed bone marrow chimaeras. A. Mixed bone marrow chimaeras were assessed for chimaerism at multiple time points. Mix D8/B6→ D8 chimaeras were divided in three groups based on inoculation ratios, mean final D8/B6 ratio in peripheral blood is indicated for each group on the x axis. B. after 11 weeks they were either NK depleted or left untreated and inoculated i.v. with a mix of fluorescence labelled RMA (test) and RBL5D^d (control) cells. Survival of target cells was analysed in spleens 18 hours after inoculation. Results are displayed as a ratio of target cells versus control cells. The figure shows one experiment with 2 mice in each NK depleted control group, and 4-6 mice in each chimaera group without NK1.1 depletion. Error bars, SD. The Mix D8/B6→ D8 groups were compared to B6→B6 group as well as among each other. Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$.



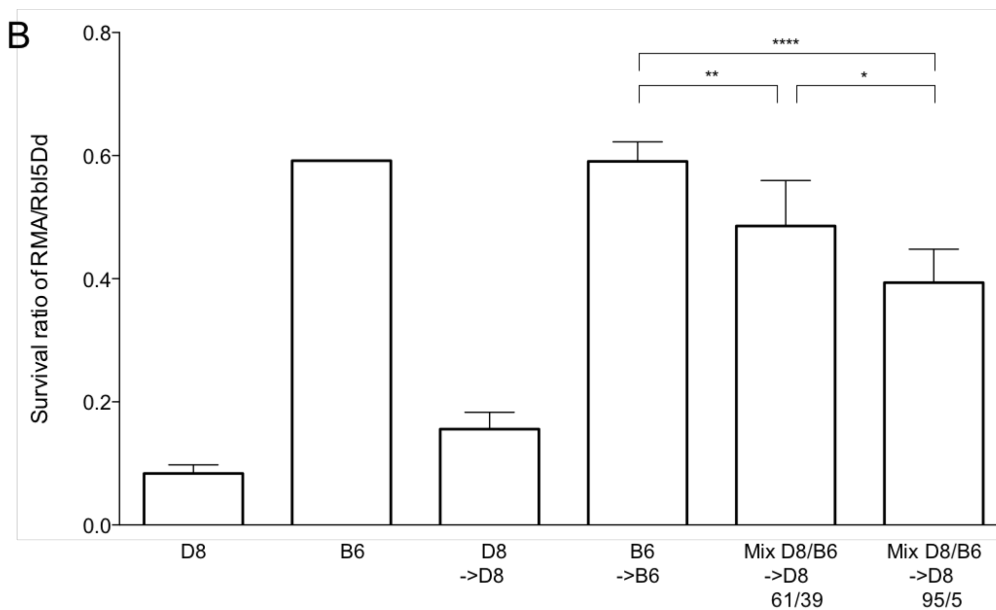


Figure 25. Analysis of RMA tumour cell rejection in stable mixed bone marrow chimaeras. A. Mixed bone marrow chimaeras were assessed for chimaerism at multiple time points. Mix D8/B6→ D8 chimaeras were divided in two groups based on inoculation ratios, mean final D8/B6 ratio in peripheral blood is indicated for each group on the x axis. B. after 10 weeks they were inoculated i.v. with a mix of fluorescence labelled RMA (test) and RBL5D[d] (control) cells. Survival of target cells was analysed in spleens 20 hours after inoculation. Results are displayed as a ratio of target cells versus control cells. The figure shows one experiment with 2 mice in each naïve group and 6 mice in each chimaera group. Error bars, SD. The Mix D8/B6→ D8 groups were compared to B6→B6 group as well with each other Statistically significant differences are denoted: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

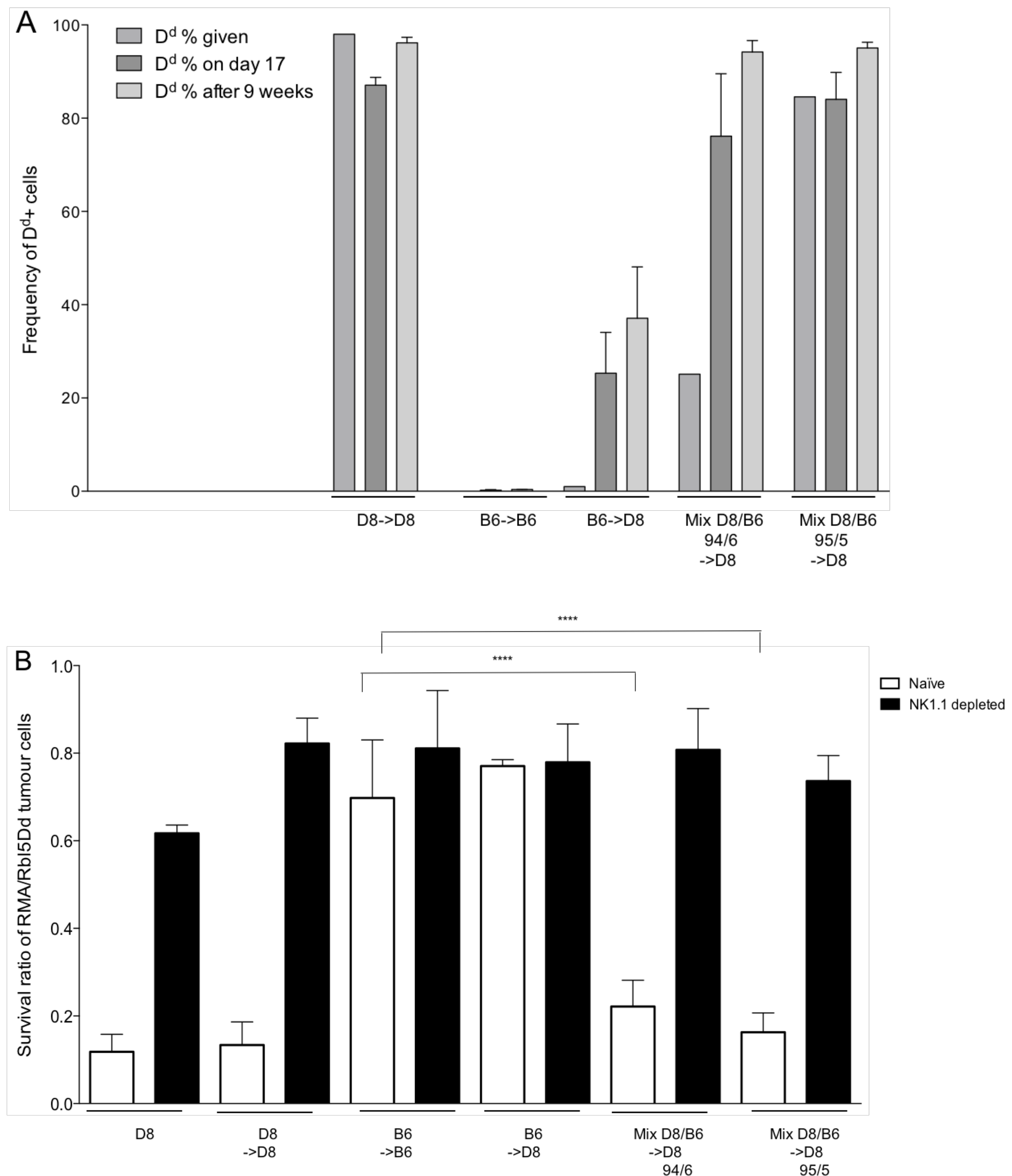


Figure 26. Analysis of RMA tumour cell rejection in unstable mixed bone marrow chimaeras. A. Mixed bone marrow chimaeras were assessed for chimaerism at multiple time points. Mix D8/B6→ D8 chimaeras were divided in two groups based on inoculation ratios, mean final D8/B6 ratio in peripheral blood is indicated for each group on the x axis. B. after 9 weeks they were either NK depleted or left untreated and inoculated i.v. with a mix of fluorescence labelled RMA (test) and RBL5D^d (control) cells. Survival of target cells was analysed in spleens 20 hours after inoculation. Results are displayed as a ratio of target cells versus control cells. The figure shows one experiment with 2 mice in each NK depleted control group, and 4 mice in each chimaera group without NK1.1 depletion. Error bars, SD. B6→D8 as well as the two Mix D8/B6→ D8 chimaeras groups were compared to B6→B6 group, and Mix D8/B6→ D8 chimaera groups were compared with each other. Statistically significant differences are denoted: *, P < 0.05; **, P < 0.01; ****, P < 0.0001.

tolerance occurred when we avoided NK cell depletion of the host, leading to the speculation that NK cells themselves are critically involved as MHC educating cells. However, there are also other possible interpretations for this observation.

From the experiments with stable mixed bone marrow chimaeras, we conclude that a low number (5%) of D^{d-} cells in the haematopoietic system reduces capacity for allele specific missing self rejection of tumour cells, but is not sufficient to induce complete NK cell tolerance to D^{d-} tumour cells. Considering that these mice showed stable chimaerism where the proportion of normal D^{d-} white blood cells did not gradually decrease with time, and therefore apparently were not rejected, these results again suggest that the quantitative regulation of NK cell tolerance differs between normal and tumour cells. In other words, it is possible to preserve some missing self reactivity to tumours even if there is complete tolerance to normal cells lacking a specific allele in a mixed MHC-I environment. Further understanding and exploration of this phenomenon may be useful in clinical settings, e.g. in haematopoietic transplantation and adoptive transfer of allogeneic (including haploidentical) NK cells. It should also be noted that strong general missing self responsiveness against MHC-I deficient RMA-S cells was seen in mixed bone marrow chimaeras (data not shown) representing a wide range of frequencies of D^{d-} negative cells. This emphasizes that NK cell anti-tumour reactivity against MHC-I deficient tumour cells can be sustained under chimaeric conditions.

Regardless of the mechanisms behind unstable chimaeras and lack of tolerance, our observations in this experiment may indirectly support the validity of our read out assay based on elimination of intravenously inoculated target cells. When the mice apparently were continuously eliminating more and more of their own normal haematopoietic D^{d-} cells, our assay revealed strong rejection of tumour cells of the same allele specific missing self phenotype. However, it should be noted that we have no formal evidence that the unstable chimaerism was caused by NK cell mediated rejection.

4.10 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In order to summarize the results of the studies in this thesis, the specific aims will first be revisited below, with a brief note on the key results and conclusions for each one of them. This will be followed by discussion and presentation of a theoretical model integrating the key results from different experimental systems in the thesis. Testable predictions for single cell analysis of NK cells are derived from this model, as well as some of its implications for immunotherapy against tumours.

Revisiting the specific aims:

- To analyse the kinetics of the effects of perturbed MHC-I sensing via NK cell inhibitory receptor blockade on missing-self reactivity.

  When MHC-I sensing by NK cells in B6 mice was perturbed by treatment with antibodies against inhibitory Ly49I/C receptors, it led to impairment of "general missing self reactivity" towards MHC-I deficient spleen cells, despite increased killing of MHC-I expressing RMA tumour cells. Both changes occurred early (16-24 hours) after inhibitory receptor blockade, and it was not possible to separate them in

kinetic studies. Efficient killing of MHC-I defective RMA-S tumour cells as well as tolerance towards normal MHC-I expressing spleen cells were preserved *(Section 4.1, Figures 4 and 5)*. The interpretation was that the inhibitory receptor blockade modulates two processes in parallel: the (re)tuning/education of NK cells (reduced responsiveness) and the effector-target interaction (increased killing).

- To further characterize and develop a previously described transgenic mouse model (DL6) encompassing mosaic expression of an MHC-I gene (D^d) [92], and in particular, to identify mice with high frequencies (>85%) of D^d expressing cells.

  The DL6 mosaic model was extended by studies of mice where the D^d transgene was present in homozygous form, and also by intercrosses to other strains. More than 1300 individual mice from different breedings were typed. In previous studies of D^d transgene heterozygous mice, the frequency of D^{d+} cells ranged between 10% and 80%, and this was not affected to a great extent in D^d transgene homozygotes. However, there was a skewed distribution towards higher frequencies of D^{d+} cells in DL6 Ly5.1 congenic mice, obtained by crossing DL6 to B6 Ly5.1 mice and subsequent intercrossing of F1 mice. These mice displayed frequencies of D^{d+} cells ranging from 30% to 97% (S*ection 4.2, Figures 6,7 and 8).*

- To develop refined *in vivo* assays for NK cell mediated rejection, to allow monitoring of three target cells simultaneously.

  It was possible to develop such assays based on staining of target cells with Violet BMQC dye and two different concentrations of CFSE dye, allowing the tracking of three types of spleen or tumour cells *in vivo*. Following kinetic analysis, we optimized the assay times to 18-24 hours for the tumour cell challenge, and to four days for the spleen cell challenge assay (*Section 4.3, Figures 11, 12, 13 and 14)*.

- To determine a quantitative threshold for NK cell tolerance to "allele specific missing self", in terms of the minimal frequency of ligand deficient cells required to induce tolerance in hosts with mosaic MHC-I expression.

  It was impossible to determine such a quantitative threshold for NK cell tolerance. All mosaic DL6 mice were completely tolerant towards normal D^{d-} spleen cells ("allele specific" missing self), even if they had a relatively high proportion of D^{d+} vs. D^{d-} cells *(Section 4.4, Figures 15, 16 and 17)*.

- To determine whether NK cell responsiveness against "general" missing self is influenced quantitatively by the proportion of cells with and without a given MHC-I ligand in mice with mosaic MHC-I expression.

  The results provided support for this hypothesis. There were varying degrees of impaired reactivity in the mosaic mice upon challenge with MHC-I defective spleen cells ("general" missing self), with a moderate inverse correlation between the number of host D^{d+} cells and the survival of these target cells. In particular, there was

an efficient rejection in the majority of mice with ≥79% D^{d+} cells (*Section 4.5, Figures 18, 19 and 20).*

- To determine whether mosaic MHC-I expression exclusively in the non-haematopoietic host environment is sufficient to influence NK cell tolerance to "allele specific" missing self and "general" missing self responsiveness.

  The results provided support for this hypothesis in the case of normal cells representing "allele specific missing self". In chimaera studies where D^{d+} bone marrow was used to reconstitute mosaic DL6 mice, there was complete tolerance towards normal D^{d-} spleen cells, indicating that a fraction of ligand deficient non-haematopoietic cells is sufficient to induce tolerance to normal cells of this phenotype. However, the general responsiveness to cells completely lacking MHC-I molecules was not significantly reduced in this situation, suggesting differences in the regulation of rejection triggered by "allele specific" missing self versus the one triggered by "general" missing self *(Section 4.6, Figure 21; Section 4.8, Figure 23)*.

- To establish a model for generation of mixed (D^{d-} vs. D^{d+}) bone marrow chimaeras with high frequencies (>85%) of D^{d+} cells, and with NK cell function maintained.

  It was possible to construct mice with stable mixed chimaerism of D^{d+} and D^{d-} haematopoietic cells in a D^{d+} host environment, using a protocol based on NK cell depletion of D8 (D^{d+}) and B6 (D^{d-}) bone marrow donors as well as of irradiated bone marrow D8 recipients *(Section 4.9, Figures, 24, 24 and 26)*. Long term stability of chimaerism was observed even when the proportion of D^{d+} vs. D^{d-} cells was in the range of 95% vs. 5% *(Section 4.9, Figures 24 and 25)*.

- To determine whether mosaic/chimaeric MHC expression has a differential influence on reactivity towards normal vs. tumour cells, with respect to allele specific as well as general missing-self rejection.

  The results from three different models supported the hypothesis on such a differential influence. In DL6 mice, tolerance towards D^{d-} RMA tumour cells representing "allele specific" missing self was not complete, in contrast to the pattern observed for D^{d-} normal cells described above. Partial rejection was observed in many mice. There was a weak inverse correlation between the frequency of D^{d+} cells and the survival of these target cells; in particular, there was a fair degree of rejection in all mice with ≥83% D^{d+} cells. There was no influence of the frequency of D^{d+} cells when rejection was assessed with MHC-I deficient RMA-S lymphoma cells; all DL6 mice rejected these cells efficiently *(Section 4.7, Figure 22)*.

  In the chimaera studies where D^{d+} bone marrow was used to reconstitute mosaic DL6 mice (i.e. full expression of D^{d} in haematopoietic cells, but mosaic expression in non-haematopoietic tissues) there was significant (but reduced) capacity for "allele specific missing self rejection" of D^{d-} RMA tumour cells *(Section 4.8, Figure 23)*.

This is in contrast to the pattern of complete tolerance observed towards normal D^{d-} spleen cells in such chimaera, as described above.

In mixed bone marrow chimaeras ($D^{d+/}D^{d-} \rightarrow D^{d+}$), there was a significant (but reduced) rejection of D^{d-} RMA tumour cells representing "allele specific" missing self. This occurred despite stable chimaerism and apparent tolerance towards normal D^{d-} cells.

The results summarized above were based on two very different approaches to perturb MHC-I self sensing (heterogeneic MHC-I environment vs. inhibitory receptor blockade), and several different experimental systems. Nevertheless, certain patterns may be discerned and it is possible to discuss many of the results within the same conceptual framework. Table 3 gives a schematic overview of key results that form the basis of such a discussion.

Complete tolerance was observed towards normal B6 target cells, in both approaches for perturbed MHC-I sensing (Table 3). This lack of reactivity was maintained in the case of inhibitory receptor blockade, where the control B6 mice were tolerant from the beginning. It was induced in the case of the mosaic DL6 mice, the main experimental model used in the thesis, where the control DL1 or D8 mice rejected B6 targets. In both experimental models, $\beta_2m^{-/-}$ targets were rejected by NK cells, albeit at reduced levels due to the perturbed MHC-I sensing. Interestingly, this reduction showed a moderate but significant correlation with the frequency of D^{d+} cells in the DL6 model. This frequency dependent, quantitative educational effect also emerged when the MHC-I expressing tumour target cells were used in the DL6 model; there was no complete tolerance towards the tumour cells in the DL6 mice (Table 3). The same trend was observed also in the mixed B6/D8 $\rightarrow$ D8 chimaeras as well as in the D8 to DL6 chimaeras (not shown in the table). On the other hand, the MHC-I deficient tumour target cells were rejected efficiently by all mice, with or without perturbed MHC-I sensing (Table 3). All of this can be interpreted within a theoretical model, which will be presented in the next section. The increased rejection of MHC-I expressing tumour cells after inhibitory receptor blockade is more complex and requires additional interpretations to fit within the model; it will be discussed further below.

Our results thus suggest that complete tolerance develops robustly even towards ligand deficient but otherwise normal host cells present only at low frequency under mosaic/chimaeric conditions. However, reactivity remains towards tumour cells of the corresponding MHC-I phenotype, which can be regarded as ''split tolerance'' (i.e. different outcome for normal and tumour cells representing allele specific missing self). The model proposed here is that the host cells deficient in the additional MHC-I molecule (D^d), modulate responsiveness of NK cells expressing inhibitory receptors only against this ligand in the education process, i.e. the D^{d-} cells are critical and dominant tutors in the education process, even if they are in minority. The frequency dependent, quantitative educational effect, revealed when using MHC-I deficient spleen cells ($\beta_2m^{-/-}$ cells) or D^{d-} tumour cells (RMA) as targets, can then be interpreted in the following way (Figure 27). The responsiveness of NK cells is tuned during the span of several encounters with other host cells, and they must thus somehow continuously "keep the books" during the education process. The education

calibrates the responsiveness for each NK cell in response to the host environment in such a way that complete tolerance is ensured, i.e. no NK cell will exceed the 'critical limit of net

Table 3. Rejection of normal and tumour target cells representing self, and 'allele-specific' or 'general' missing self

Target cells → / Mouse ↓	B6	$B_2m^{-/-}$	RMA	RMA-S
D8 and DL1	++	+++	+++	+++++
DL6 (high frequency D^{d+})	—	++	++	+++++
DL6 (low frequency D^{d+})	—	+	+	+++++
B6	—	++	+	+++++
B6 $F(ab')^2$ inhibitory receptor blockade	—	+	++	+++++

The number of '+' signs reflects the strength of rejection. The '—' sign denotes no rejection.

signalling required for killing' in any encounter with such normal host cells. This is achieved by a reduction of responsiveness in certain NK cell subsets with inhibitory receptors for D^d molecules (but not for other MHC-I molecules) in DL6 mice, correlating with the number of D^{d-} cells in the environment. The reduced responsiveness cannot be read out with normal D^{d-} cells as targets, because all NK cells fall below the critical limit of net signalling required for killing in the context of those (K^b D^b expressing) cells; complete tolerance is registered. The situation is different for tumour cells of the D^{d-} phenotype, because these are assumed to possess more activating ligands than normal cells, and also for MHC-I deficient normal cells, because these lack MHC-I ligands for other inhibitory NK cell receptors. These targets, therefore, fall within a window where the quantitative influence in the education process can be read out in different DL6 mice. However, RMA-S tumour cells with high expression of activating ligands and no MHC-I molecules, also fall outside of this window but in the opposite way compared to normal B6 spleen cells; these tumour targets are already highly sensitive to NK cells that have seen no D^d ligands (e.g. in B6 mice). Despite reduced general missing self responsiveness in DL6 mice, RMA-S cells are so sensitive that they are still efficiently killed.

A similar interpretation can be applied to the results observed in the model based on inhibitory receptor blockade, although this experimental model did not allow us to correlate quantitative effects. In the paper where these results have been published, we proposed that the reduced inhibitory input caused by receptor blockade adapted the targeted NK cells ''to

the signals received from normal cells in the environment; this can explain the reduced rejection of normal MHC class I⁻ cells, as well as the robust tolerance observed against normal MHC class I–expressing cells after inhibitory receptor blockade'' [222] While the adapted NK cells show reduced responsiveness towards MHC-I deficient healthy cells, this is not observed for the MHC-I expressing tumour cells, which lose the advantage of MHC-I

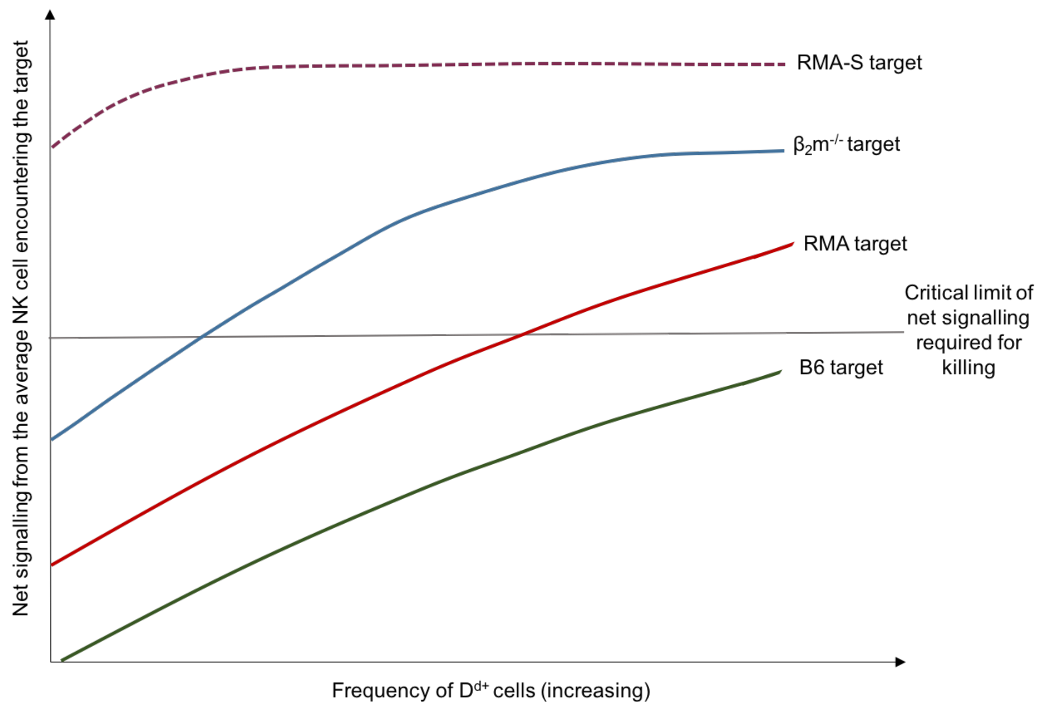


Figure 27. Net signalling from the average NK cell encountering the target, as a function of increasing frequencies of D^{d+} cells.

expression in the effector-target interaction due to the receptor blockade. These are, therefore, killed more efficiently in the treated mice, which explains the difference observed when RMA tumour cells are used as targets in the inhibitory receptor blockade vs. the DL6 model (Table 3).

There are thus, two opposing effects of the antibody treatment, the first on education (down-tuning) and the second on the effector target interaction (increased killing) (Figure 28). The normal MHC class I deficient target cells reveal only the first effect, while the second effect dominates when MHC-I expressing RMA tumour cells are used. None of the effects are revealed with the normal MHC-I expressing cells in the *in vivo* rejection assay, since the down-tuning leads to complete tolerance towards these cells. The model proposes that the difference between MHC-I expressing tumour and normal cells is caused by higher expression of activating ligands on the former. Even if the targeted NK cells have been rendered less responsive, they still get sufficient activating net signalling when encountering the tumour cells, while the normal cells fall below this threshold. Finally, the RMA-S tumour cells also do not reveal any of the effects, because their impaired MHC expression and high

expression of activating ligands result in strong activating net signalling to NK cells, well above the critical threshold, in the presence as well as absence of inhibitory receptor blockade. Hence, strong rejection is observed in all situations (Table 3).

This proposed interpretation suggests that the inhibitory receptor blockade also causes reduced NK cell responsiveness against the tumour cells, even if this is not revealed in the net effect observed in our experimental model based on RMA tumour cells. Other tumour cells may give different results, depending on their levels of activating ligands and other factors.

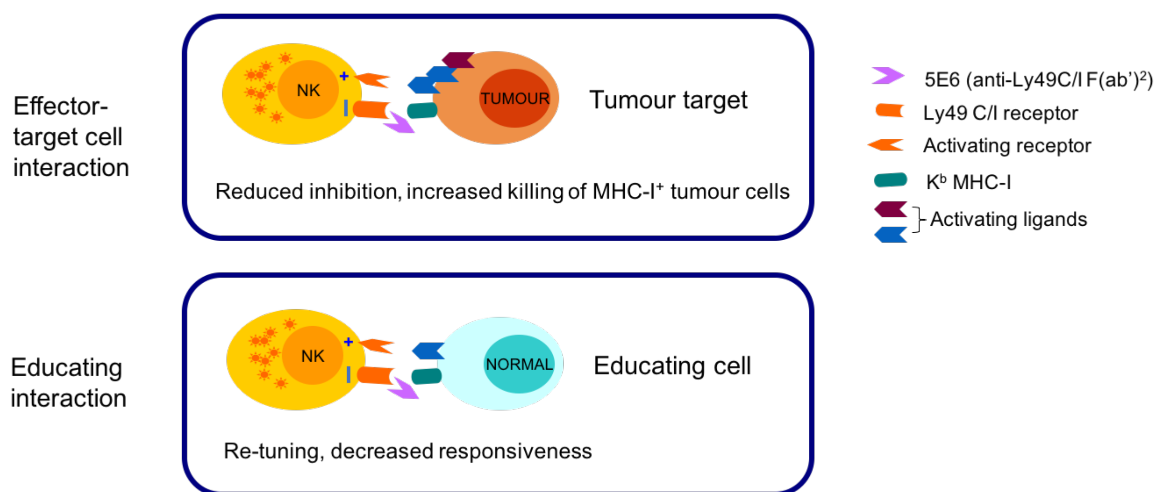


Figure 28. Two potential effects of inhibitory receptor blockade, influencing the effector target interaction or education.

Indeed, Carlsten et al. observed reduced *in vitro* responses to K562 tumour cells by NK cells from smouldering multiple myeloma patients, treated with "KIR blockade" antibodies [223]. In future development of inhibitory receptor blockade, it may, therefore, be important to develop strategies that maximizes the influence on the effector target interaction, and minimize the influence on the education process. This may be achieved by intermittent rather than continuous blockade, and by combination with other antibodies or cytokines that increase the responsiveness of NK cells.

If the model above is correct, it is possible to infer clear and testable predictions concerning single cell features of NK cells in the DL6 mice, which link the frequency of D^{d+} cells to the function of NK cells with inhibitory receptors for D^d. A schematic overview is presented in Table 4, where the three first columns represent NK cells with at least i) one receptor for K^b D^b (but none for D^d), ii) one receptor for K^b D^b and one for D^d, iii) one receptor for D^d (but none for K^b D^b). Since the two first subsets possess receptors for ligands which are expressed by all cells in all the mice, there are only marginal predicted differences for the single cell responsiveness (e.g. CD107α/IFN-γ expression after stimulation with antibodies against activating receptors) between the mice. It is the third subset, which only possesses receptors

for D^d, that is critical and mainly responsible for the differences that we have observed in *in vivo* experiments.

Any molecular determinants of MHC-I dependent education in these cells would also be predicted to correlate with the frequency of D^d expressing cells in the DL6 mice. So far, no such determinants have been identified. It is still unclear how education alters the signalling that sets the threshold for effector responsiveness. However, it appears that high expression of KLRG1 may be maintained as a downstream consequence of education. DNAM-I shows a similar pattern, except in NK cells that express NKG2A. It has recently been shown that DNAM-1 is turned on early during NK cell maturation in the bone marrow, and that the expression of this receptor gradually fades in all $NKG2A^-$ NK cells as they mature, except in those subsets possessing Ly49 receptors for host MHC-I molecules, i.e. the cells that become educated for high responsiveness (Wagner et al. Nature Comm, in press, 2017). It is, therefore, predicted that the expression of DNAM-1 as well as of KLRG1, should correlate with the frequency of D^{d+} cells in DL6 mice.

Table 4. Predictions by the proposed model, regarding NK cell features in single cell assays

Receptors → Mouse ↓	At least one Ly49r for K^b or D^{b*1}, but no receptor for D^{d*2}	At least one Ly49r receptor for K^b or D^b, and at least one receptor for D^d	No receptors for K^b or D^b, and at least one receptor for D^d	
	Responsiveness	Responsiveness	Responsiveness	Surface expression of KLRG1 and DNAM-1*3
D8 and DL1	+++	++++	+++	++++
DL6 (high frequency D^{d+})	+++	++++	++	+++
DL6 (low frequency D^{d+})	+++	+++	+	++
B6	+++	+++	—	+

*1 Ly49 receptors for K^b or D^b include Ly49C, I
*2 Receptors for D^d include Ly49A and G2
*3 DNAM-I expression on $NKG2A^-$ cells
The number of '+' signs reflects the strength of rejection. The '—' sign denotes no rejection.

When testing these predictions, it may be important to bear in mind that despite the observed correlations between frequency of D^{d+} cells and capacity for "allele specific" or "general" missing self reactivity, there was considerable variability in the rejection by individual DL6 mice, across the entire range of mosaic MHC-I expression. We do not know the reason for this variability. It may be caused by factors unrelated to NK cell education, such as nutritional, hormonal, microbial factors. It cannot be excluded that the variability reflects actual differences in education, caused by additional receptor-ligand interactions, that are superimposed on or integrated with the effects of D^d molecules on inhibitory NK cell Ly49 receptors. One possibility is that NK cells expressing the activating receptor Ly49D, known to bind to D^d, are also affected by the mosaic composition in the DL6 mice, but in the opposite manner to NK cells expressing inhibitory D^d receptors. This can actually be dissected experimentally by including staining for Ly49D expressing cells in scheme,

allowing gating on Ly49D single receptor expressing cells, as well as cells expressing Ly49A or Ly49G2 with or without Ly49D.

"Split tolerance" towards normal and tumour cells with the same MHC-I phenotype may be harnessed in immunotherapy, where heterogenous MHC-I expression occurs in the haematopoietic compartment, e.g. in haematopoietic transplantation or adoptive transfer of allogeneic NK cells. Transplantation of haploidentical or partially HLA-matched NK cells has been associated with reduced risk of GVHD and increased potential for GvL effects [135, 234]. In other words, while exhibiting anti-tumour effects, these donor NK cells show tolerance towards normal cells expressing partially matching HLA, in the host. This is in line with the model presented here, since NK cells educated by KIR interactions with donor cells and lacking inhibitory KIRs for the recipient HLA ligands, will be tuned to tolerate normal recipient cells, but still kill tumour cells. Donor NK cells having inhibitory KIRs for the host HLA will also not attack normal recipient cells, but may kill tumour cells due to the same reason as mentioned above i.e. increased expression of activating ligands. To exploit this situation clinically, the gap in 'split tolerance' may be widened enough so that the anti-tumour activity is increased while the reactivity towards normal cells may be reduced even further. It should finally be noted that NK cells lacking KIR for both donor and recipient HLA-ligands, i.e. non-MHC-I educated cells, may mediate anti-tumour activity under certain conditions. Evidence arguing for the efficacy of such cells has been provided in a study of patients receiving immunotherapy with IL-2 and histamine dihydrochloride (Bernson et al. Abstract, 16th Annual meeting of the Society for Natural Immunity NK2106, Taormina, Italy).

An additional suggestion from the model is that one should transplant, transfer or strive to maintain as high frequency of donor haematopoietic NK cells as possible, in agreement with observations made in certain studies [146, 157, 235]. It has also been shown in a mouse chimaera study that tolerance may be induced in NK cells by either the haematopoietic or the non-haematopoietic tissue, with both situations favouring tolerance to the 'ligand negative' cell type *in vivo* [211]. However, it was observed that while tolerance induced by the haematopoietic tissue could be broken by infection or cytokine induced inflammation, this was not the case for that induced by the non-haematopoietic tissue, showing its stronger impact. This has been attributed to the presence of distinct educating mechanisms for NK cells by these tissue types. In a clinical situation, this may mean that reconstitution of a patient's haematopoietic system with a donor graft lacking one or more HLA alleles may end up in breaking of tolerance towards the donor cells in case of an infection, and cause graft rejection. On the other hand, based on the model presented above and other studies, it may be recommended to select HSCT donors based on expression of several different KIR ligands, so that the NK cells without a KIR ligand in the host may exert their reactivity more effectively towards tumour cells. Quantitative and dynamic influences on NK cell education may prove important in the clinical use of NK cells, and this area should be exploited in further studies.

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6 ACKNOWLEDGEMENTS

I am at a loss for the right words to express my gratitude.

I would like to sincerely thank my supervisor Klas Kärre for sharing practical details, illuminating discussions and wise interpretations of complex phenomena. It has been very inspiring.

I am grateful to my co-supervisor Maria Johansson for always being available, for the support in practical work and for the detailed and prompt feedback, especially during writing this thesis.

I thank my co-supervisor Hanna Brauner for the enthusiasm, positivity and support during writing this thesis.

Together, I thank you for the enormous help in finishing this thesis. It would not have been possible otherwise. I have learnt a lot from you, especially in these last few months, about science as well as life in general. Thanks to my co-supervisor Laksmikanth Tadepally for getting me started in the lab, to my collaborators, past and present members of the Kärre group and associated groups for all their help, interesting discussions, meetings, and the nice Christmas parties!

A big thank you to all the people who took interest in my project, Kenth Andersson and others in the animal department for always accommodating me, and my follow-up seminar and half time committee members especially Benedict Chambers. Many thanks to all MTC colleagues, departments including facilities and services in MTC, my course leaders and the funding agencies, especially HEC Pakistan.

Thanks to my mentor Lalit Kumar for always reminding me of the big picture, to Susanna Cardell and Vivianne Malmström for their valuable suggestions for improving this thesis.

Thanks to my wonderful friends and colleagues in various departments at KI and KS especially CCK and FRH, at other universities, my conference-partners in Glasgow and Heidelberg, my course mates in the animal science course at KI, immunology course in Sorrento, pain studies course in Minnesota and infectious diseases course in Ghana.

Grateful beyond words to my amazing friends from the Strix corridor (home), from Jägargatan, from Stockholm, other cities, and from outside academia. It has been a pleasure.

To all the people with whom I shared nice company, talks, ideas, travels, meals, and the ups and downs, you are and will be, in my fond thoughts. Heartfelt thanks to the unsung heroes.

I am deeply indebted to my family and extended family in Vienna, Minnesota and Pakistan, who have always had my back through thick and thin, shared my enthusiasm and encouraged me to pursue what I wanted, how I wanted. Bari Ammi, Abba Ji, Ammi, Abbu, Abbi,

Kishwar Ammi, Fiaz Mamoon, Billoo Mamoon, Jawad, Hammad and Jiya, I cannot appreciate that enough, Thank you!

I thank God for the opportunities and perseverance.

Finally, one goes out to the mice used, with a hope that this might make even a small difference!

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