The natural killer cell receptor NKp30 and its cancer cell ligand B7H6

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PhD thesis

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Section for Anatomy

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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>BAG-6</td>
<td>Bcl-2-associated athanogene 6</td>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
<td>ICOS</td>
<td>Inducible T-cell costimulatory</td>
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<tr>
<td>BDI</td>
<td>Bright detail intensity</td>
<td>ICOS-L</td>
<td>ICOS ligand</td>
</tr>
<tr>
<td>BiTE</td>
<td>Bispecific T cell engager</td>
<td>iDC</td>
<td>Immature dendritic cell</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
<td>IFC</td>
<td>Imaging flow cytometry</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin-like receptor</td>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte associated antigen 4</td>
<td>ILC</td>
<td>Innate lymphoid cell</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>DAP10/12</td>
<td>DNAX-activating protein of 10/12 kDa</td>
<td>ILTAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>DBL</td>
<td>Duffy binding-like</td>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
<td>KIR</td>
<td>Killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>ECTV</td>
<td>Ectromelia mousepox virus</td>
<td>LILR</td>
<td>Leukocyte immunoglobulin-like receptor</td>
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<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
<td>LRC</td>
<td>Leukocyte receptor gene complex</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
<td>MCMV</td>
<td>Mouse cytomegalovirus</td>
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<tr>
<td>LTI</td>
<td>Lymphoid tissue inducer</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>mDC</td>
<td>Mature dendritic cell</td>
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<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cells</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
<td></td>
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<tr>
<td>MICA/B</td>
<td>MHC class I polypeptide-related sequence A/B</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>NCR</td>
<td>Natural cytotoxicity receptor</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NKC</td>
<td>Natural killer complex</td>
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<tr>
<td>PAMP</td>
<td>Pattern-associated molecular pattern</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD</td>
<td>Programmed death</td>
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<td>PD-L1/2</td>
<td>PD-ligand 1/2</td>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
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<tr>
<td>PfEMP</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<tr>
<td>ScFv</td>
<td>Single chain variable fragment</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;1/2</td>
<td>T helper cell 1/2</td>
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<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>TMIGD</td>
<td>Transmembrane And Immunoglobulin Domain Containing 2</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>T regulatory cells</td>
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<tr>
<td>ULBP</td>
<td>UL16 binding protein</td>
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<td>VV</td>
<td>Vaccinia virus</td>
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<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Paper II.

Paper III.
INTRODUCTION

The immune system is the summation of cells, molecules and biological processes that enable the host to recognize pathogens, elicit adequate protective mechanisms that will eliminate the threat and finally terminate the response to prevent autoimmune reactions.

The immune system is by convention divided into two main branches; the innate and the adaptive immune system. The former comprises soluble factors and several different cell types that collectively make up the host’s first line defense system. It is present from birth and does not change considerably throughout life. Although slower to respond than the innate system, the adaptive immune response provides the host with immunological specificity as well as long-lasting memory and is developed during the lifetime of an individual.

The innate immune system

The innate immune system is found in all organisms, representing an ancient form of host protection and mounts an immediate response towards invading pathogens. The skin and mucosal surfaces represent physical barriers and are main sites of contact with microorganisms. When intact, the epithelia do not allow passage of pathogens due to the tight junctions that connect the cells together. Other defense mechanisms, like secretion of mucus, enzymes, antibacterial peptides and low pH, ensure the destruction of pathogens. Commensal bacteria also colonize the epithelial surfaces, where they compete for nutrients and secrete antimicrobial peptides, thus inhibiting growth of pathogenic microorganisms. Should the pathogen breach the physical barrier, an arsenal of immune mechanisms will limit its invasion. These include complement activation, inflammation and cellular responses. For instance, in bacterial infection, macrophages are abundant in the sub-mucosal layers, and will upon encounter with a pathogen elicit an inflammatory response that will recruit other immune cells to the site of infection. Cytokines secreted by the macrophage will also lead to maturation of dendritic cells, differentiate monocytes into macrophages which, along with recruited neutrophils, will phagocytose the bacteria. Natural killer cells also belong to the innate immune system, and can eliminate virus-infected or malignant cells, which will be discussed in greater detail below. The innate immune system will handle a majority of infections before most pathogens can cause a disease. Infections that are not controlled by the first line defense can be defeated by the adaptive immune system.

The adaptive immune system

In jawed vertebrates, the major effector cells of the adaptive immune system are B and T lymphocytes. Early in maturation of these cells, somatic recombination of a limited number of gene segments encoding the B and T cell receptor (BCR, TCR respectively) results in a large repertoire of antigen specific receptors, that can recognize (almost)
any molecular pattern. Thus; single cells are specialized to target a specific antigen, and hence only a few clones are able to elicit a response towards a particular pathogen. Following primary infection, the engagement of a specific B or T cell receptor triggers proliferation of the B or T cell, i.e. clonal expansion, followed by differentiation into effector cells and generation of immunological memory. This is a comprehensive process as it requires cell division of one pathogen-specific cell to become an army big enough to manage the invader. This is the reason why the adaptive response is slower than the innate. However, upon re-encounter with the same pathogen, pathogen-specific memory cells respond more rapidly, leading to a faster clearance of the threat.

How does the immune system recognize infection and cancer?
The immune system constantly faces the challenge of recognizing infectious agents, but can also detect cell changes in cancer development. When a pathogen crosses the initial host barriers it must avoid recognition in order to survive. Microorganisms have short generation times. This provides them with a great advantage as it enables them to evolve rapidly by continuing acquired mutations that let them escape host immunity. Tumor cells also evolve mechanisms to escape immune recognition (to be discussed later). Despite these mechanisms, the plasticity of the immune system allows it to keep up in this arms race against external and internal threats.

Infection
Infection can be described as an invasion by a pathogenic microorganism, resulting in anything from symptom-free, subclinical disease, to tissue damage at site of infection, systemic disease and even death of the host. A successful pathogen is able to cross the initial host protective barriers, establish itself at the site of entry and evade the host immune response. Microbial infection and tissue damage are recognized by a group of receptors called pattern recognition receptors (PRRs). These are expressed by innate immune cells such as macrophages and dendritic cells, but also to some extent non-immune cells like fibroblasts, epithelial and endothelial cells (1). The PRRs recognize conserved molecular patterns that are unique to the microbe and not expressed by the host, collectively known as pathogen associated molecular patterns (PAMP). The PAMPs are essential to pathogen virulence, and could not easily be changed by mutation. Thus, PRR recognition of these exact patterns is the result of strong selective pressure in the arms race between host and microbe. Several different families of PRRs exist. They have different structure, function and cellular location, shaped by evolution to target the different strategies of pathogenic invasion. The Toll-like receptors (TLRs) and C-type lectin like receptors (CLRs) are transmembrane proteins, whereas Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) are expressed in the cytoplasm (2, 3). These groups have several members recognizing the diverse panel of microbial ligands. It has also become apparent that some PRRs are able to recognize endogenous proteins expressed by
stressed or dying host cells, known as damage associated molecular patterns (DAMPs). Engagement of most of the PRRs results in release of cytokines that will initiate inflammation. PRR signaling is also important for antigen uptake and presentation by antigen presenting cells, thereby bridging the immune response between the innate and adaptive branches.

Despite the fact that our immune system seems well equipped with strategies to protect us from infections, some viruses and bacteria still successfully invade and survive the immune response. One such example is cytomegalovirus (CMV) that has dedicated a large proportion of its genome to genes that counteract the host immune system. Belonging to the herpes family, CMV is widespread in mammals, and a majority of the adult human population is infected. In healthy individuals, CMV is usually harmless and asymptomatic, but can in immunocompromised patients become life-threatening (4). After the immune system repels the initial acute infection, the virus enters a state of latency where few genes are transcribed and no viral progeny is generated. The virus remains dormant in myeloid cells, interrupted by the occasional reactivation where virions are released into the bloodstream and symptoms become present, especially in immunocompromised patients. CMV triggers most branches of the immune system. The innate response to the initial entering of CMV into a host cell is partly mediated by TLR2, which recognizes the envelope glycoproteins gB and gH (5, 6), leading to secretion of inflammatory cytokines. Humoral immunity also plays a protective role, manifested by antibodies towards viral proteins (pp65 and pp150) as well as gB and gH (7, 8).

CMV infected cells are recognized and killed by T cells. Their importance has been seen in both murine MCMV models as well as in patients undergoing bone marrow transplantation, where HCMV specific CD8+ T cells confer an important immune response to the virus. HCMV seropositive patients have CD8+ T cells that respond to peptides derived from virally encoded proteins such as pp65 and immediate early protein (IE) (reviewed in (9)). The virus employs several strategies to evade detection by CD8+ T cells, by interfering with MHC class I processing and presentation pathways. Examples include viral genes encoding proteins that degrade class I heavy chains (US2 and US11), retention of MHC I-peptide complexes in the endoplasmatic reticulum (ER) (US3) or blocking of peptide translocation into the ER (US6). CD4+ T cells also play a protective role against CMV. CMV strategies to avoid CD4+ T cell detection are similar to those against CD8+ T cells, targeting the processing of MHC class II. During latency, the virus has also been shown to express a homologue of the immunoregulatory IL-10 that downregulates expression of MHC class I and II, and inhibit the production of inflammatory cytokines.

NK cells also play a vital role in protection against CMV infection and individuals lacking NK cells suffer severe, recurrent infections. The antiviral response of NK cells
includes cytokine secretion leading to activation of other arms of the immune system, and killing of the infected cell by release of cytotoxic granules that contain perforin and granzymes. NK cells express inhibitory receptors that recognize MHC class I. The CMV-induced downregulation of MHC class I to evade CD8$^+$ T cell recognition leads to activation of NK cell cytotoxicity due to absence of inhibitory signals (also known as missing self, described later). However, CMV has developed strategies to circumvent NK recognition as well. A well-known example in mice is m157, an MHC class I homologue expressed by certain MCMV strains. This molecule binds inhibitory Ly49I, preventing the NK cell from killing the infected cell. However, m157 also engages the activating Ly49H. Thus an Ly49I$^+$/Ly49H$^-$ mouse strain is not able to mount an NK cell response towards a CMV strain expressing m157, whereas Ly49H$^+$ strains readily fight off infection (10). It is conceivable that the emergence of m157 has driven the evolution of Ly49H from the inhibitory Ly49I by gene duplication. Furthermore, Ly49H-m157 engagement exerts a strong evolutionary pressure on the virus, presumably leading to the high variability in m157 expression between MCMV strains.

In humans, a similar decoy molecule has been described. LILR-1 is an inhibitory NK cell receptor that binds all groups of classical HLA molecules. To compensate for the loss of MHC class I, CMV expresses a decoy molecule, UL18, which binds LIR-1, inhibiting the NK cell.

Cancer
The immune system does not only deal with extrinsic threats, but also plays a major role in detection and elimination of malignant cells. The importance of immune protection against cancer was first demonstrated in the 1890s by William Coley, as he observed that cancer patients with post-operative bacterial infections had a longer lifespan than those without, and that inoculating patients with streptococcal cultures had anti-tumor effects in some patients with sarcoma and lymphoma (11). The main immune cell effectors that recognize and eliminate tumor cells are NK cells and cytotoxic CD8$^+$ T cells. In the early stages of tumor development, these cells exert a strong selective pressure on the malignant cells by killing the more immunogenic cancer cells. Macrophages of the pro-inflammatory M1 phenotype also participate at this stage. This can then select for the survival and proliferation of a tumor cell population that is less immunogenic and less susceptible to attack by the immune system. As the initial tumoricidal effector response decreases due to reduced immunogenicity, the neoplastic tissue grows, disseminates and evolves strategies to induce peripheral immune tolerance.

The recognition of tumor development by the immune system has been termed immunosurveillance. The immune cells recognize tumor-associated molecules such as stress-induced ligands or neoantigens. Stress-induced ligands are molecules not normally expressed by healthy cells that can become upregulated as a result of
malignant transformation. Examples include RAE-1 and H60 in mice, and MICA/B and possibly also B7H6 in human (12-16). They bind activating receptors on NK cells resulting in the release of cytotoxic granules containing perforin and granzymes that induce apoptosis in the tumor cells. NK cells can be recruited to and eliminate established solid tumors that produce IL-15 (17), but the role of NK cells in solid tumors is less understood. The second node of cancer immunosurveillance is the recognition of neoantigens - or altered self. These are peptides presented by tumor cells derived from somatically mutated proteins as a result of tumor development. The location of the mutation may affect how the peptide is anchored on the MHC, thus affecting affinity and binding to the MHC and hence the interaction with the TCR. If the mutation is at the interface between peptide/MHC and the TCR, the mutated peptide may be recognized by a naïve T-cell, as these neoepitopes are not subject to thymic selection and central tolerance. The first neoantigens to be recognized were in melanoma patients, and it was shown that the primary antitumor response was driven by neoantigen-specific T cells (18-20). Furthermore, CD4+ T cells initiating a Th1-mediated anti-tumor response by secretion of IFNγ, IL-2 and TNFα will promote the activation of cytotoxic T cells, NK cells and macrophages. The presence of these effector cells or their cytokines in tumors has been associated with favorable outcomes in several diseases.

Immune evasion strategies by tumors are numerous. The genetic instability and ability to rapidly divide enables tumor cells to reduce their immunogenicity allowing them to escape detection by immune cells. A well-established mechanism that tumors use to escape immune recognition is by interfering with antigen presentation. Cancer cells down-regulate MHC class I for instance by affecting the peptide transporter protein TAP, thus evading recognition by cytotoxic T cells (21). Immune suppression in the tumor microenvironment by exerting control over T regulatory cells (Tregs) and other immunosuppressive cells is also a major strategy of tumor immune escape. Tumor cells secrete chemokines that attract Tregs, and numerous studies have shown that Tregs resident in tumors are more suppressive than their peripheral counterparts (22, 23). Tumor cells may also produce TGF-β that converts surrounding CD4+ T cells to Tregs (24). Another example is myeloid-derived suppressor cells (MDSCs) that contribute, together with DCs and certain macrophage phenotypes, to a tumor-beneficial inflammatory environment that promotes tumor initiation, angiogenesis and metastasis (25). Thus, the tumor microenvironment is of great importance, as many immunomodulatory cytokines cripple the cytolytic responses of T and NK cells. TGF-β is one example (26). T cell anergy is also seen as a result of engagement of inhibitory molecules, such as programmed death ligand 1 (PD-L1) upregulated on tumor cells (27). The discovery of tumor involvement in co-stimulation of T cells has led to great therapeutic advances which will be discussed later. It has also been shown that tumor cells selectively shed ligands for activating receptors, such as B7H6 and
NKG2D-L (28-30). The reduced surface expression of these ligands leads to poorer recognition by immune cells, and additionally, the shedded ectodomain will bind the receptors and block their binding site, thus preventing them from engaging other intact ligands.

**NK cells**

As discussed above, NK cells play an important role in host defense against viral infections as well as tumor development. They shape immune responses by secreting chemokines and cytokines, but first and foremost, they are known for their ability to spontaneously recognize and kill a virus-infected or malignant cell without prior sensitization. It was by this characteristic that NK cells were initially discovered, as it was observed that a lymphocyte subset distinct from B and T cells specifically lysed both syngeneic and allogeneic virus-induced leukemia cells without any priming (31-33). This spontaneous killing of target cells became known as natural cytotoxicity, giving rise to the name “natural killer” to the cells responsible.

NK cells were originally thought only to develop in the bone marrow, but recent evidence has shown that progenitor cells can also develop and mature in tonsils, lymph nodes and spleen (34). In humans, CD56^{bright}/CD16^{low} NK cells readily produce cytokines and constitute the largest NK subset in tonsils and lymph nodes. They do however lack the strong cytotoxic ability of their CD56^{dim}/CD16^{positive} counterparts. The latter subset expresses high amounts of perforin and make up 90% of NK cells in peripheral blood and spleen. NK cells make up 5-15% of all lymphocytes in the blood, and are present to varying degrees in different tissues. As sentinels of the immune system, their location is not limited to lymphoid organs. Rather, NK cells are found in various non-lymphoid tissues as well, such as liver, gut, kidney, uterus and lung (35).

In order for NK cells to become fully functional, it has been proposed that they undergo a process called “licensing” during development. This model describes the process where NK cells are required to express inhibitory receptors to achieve functional competence. If they are not able to receive inhibitory signals, or the activating signals override the inhibitory, the NK cell will remain hyporesponsive (36). “Silencing” is an alternative model, which suggests that mature NK cells become hyporesponsive due to persistent activating signals, uninterrupted by inhibition (37).

**NK cell effector functions**

NK cells recognize various states of target cells; the recognition of *self* through MHC class I binding receptors, *missing self* as a result of lack of MHC class I expression, *induced self* - a result of upregulation of ligands for activating receptors or *altered self* resulting from viral infection or malignant transformation (38). The specificity for self MHC became clear in allotransplantation studies in mice and rat where NK cells were able to kill MHC disparate bone marrow-derived cells (39, 40). The F1 hybrid
resistance model confirmed this, as a heterozygous offspring of two MHC disparate parents rejected grafts from the parental strains (41, 42). These observations could largely be explained by the missing-self hypothesis (43, 44). Accordingly, NK cells are actively inhibited by the presence of MHC class I on normal, syngeneic cells. Thus, when encountering a cell with reduced/absent surface expression of self MHC class I, the inhibitory signals are abrogated and the NK cell becomes activated. With the identification of the MHC-specific killer cell immunoglobulin-like receptors (KIR) in humans and Ly49 in mice the molecular basis for MHC recognition as well as missing self was determined (45). Although some of the results observed in the rodent alloreactive experiments could not be explained by the missing self hypothesis, as they suggested the existence of activating allospecific NK cell receptors (46, 47), it was not until the cloning of activating Ly49s and KIR it was fully accepted that the activity of NK cells is regulated by both stimulatory and inhibitory receptors (48, 49).

Recognition of missing, induced or altered self may result in activation of the NK cell, provided the balance of intrinsic signals shifts from inhibition towards activation (Fig. 1). NK cells may also be stimulated at the site of inflammation by resident macrophages secreting IL-12, IL-15 and IL-18 (35). Once activated, the two main effector functions of NK cells are either to induce apoptosis in the target cell, or to release cytokines, primarily of the Th1 type (IFNγ, TNFα, GM-CSF). These cytokines will activate T cells, DCs, macrophages and neutrophils. NK cells can also produce

**Figure 1.** Target recognition by NK cells. An NK cell response is regulated by a balance of activating and inhibitory receptors.
chemokines such as macrophage inflammatory protein (MIP)-1α and -1β, lymphotoxin and IL-8 that will recruit other immune cells to the inflamed tissue (34). The death of target cells requires formation of an immunological synapse at the site of contact between the NK cell and its target. The immunological synapse is a dynamic and organized structure, that was originally described in T cell-APC interaction (50). The result of synapse formation during activation is a specific polarization of receptors and cytotoxic granules just beneath the center of the synapse mediated by cytoskeletal rearrangements (51, 52). Once released, perforin and granzymes create pores in the target cell membrane and activate caspases that results in initiation of apoptosis (53, 54).

Cytotoxicity can also be mediated by the death receptors FAS and TRAIL whose ligands are expressed by NK cells. The secretion of IFNγ by NK cells induces the expression of these receptors on the target cell, and once engaged by their corresponding ligands on NK cells, the death receptors initiate apoptosis. NK cell also express CD16 (FcIIIγRA), a receptor that binds the Fc part of IgG. Thus, upon encounter with antibody-coated targets, the NK cell kills the target in a process called antibody-dependent cell-mediated cytotoxicity (ADCC) (55, 56).

**NK cell receptor families**

Inhibitory NK cell receptors contain an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic tail. Upon ligand binding and phosphorylation, ITIMs recruit tyrosine phosphatases (SHP-1 or SHP-2) or the inositol phosphatase SHIP, which acts to de-phosphorylate signaling intermediates in the pathways that activate NK cell effector functions. Activating receptors usually have cytoplasmic tails with no intrinsic signaling function, but rather rely on association with adaptor molecules that contain immunoreceptor tyrosine-based activation motifs (ITAMs). Such adaptor molecules include FcεRIγ, CD3ζ, DAP10 and DAP12. Activating NK cell receptors bind these adaptor molecules through oppositely charged amino acids in their respective transmembrane regions. Upon phosphorylation, ITAMs will recruit and activate the tyrosine kinase Syk, which will phosphorylate several signaling intermediates leading to release of cytotoxic granules or cytokine. DAP10 contains a YXNM motif that recruits PI3K and Grb2, and thus induces NK cell effector functions by partly different downstream pathways (reviewed in (57)). Most NK cell receptors are localized within two major chromosomal regions. The leukocyte receptor gene complex (LRC) encodes proteins belonging to the immunoglobulin-like superfamily, whereas the natural killer complex (NKC) encodes C-type lectin-like receptors. Both clusters are present in all mammalian species investigated, but there is substantial species-specific expansion of the different gene families.
The immunoglobulin superfamily

The concept of the immunoglobulin superfamily (IgSF) originated from observations that domains within a variety of protein families share sequence similarities and a basic three-dimensional structure with the protein domains found in the immunoglobulin variable and constant regions. The IgSF constitutes the largest superfamily in the human genome, and IgSF domains are the most common domains in leukocyte membrane proteins (58). IgSF receptors are thought to have evolved from a single ancestral gene by gene duplication and exon shuffling (59, 60). This is supported by a “module” gene arrangement where most Ig-like domains are usually encoded by single exons, with a phase I intron/exon boundary, where splicing occurs after the first nucleotide of the codon. A typical IgSF domain consists of 75-100 amino acid residues and has a sandwich-like structure formed by two $\beta$-sheets made of antiparallel $\beta$-strands (Fig.2). The strands are named A-G based on their order of appearance in the sequence. Within the B and F strands are two cysteine residues, separated by approximately 55-70 amino acids, which form a disulfide bridge between the two sheets. The traditional classification of Ig-like domains divides the structures into V-, C1- and C2-set Ig folds, but there are a great number of variations within these. The core of the V-set fold is made of $\beta$-strands A, B, E and D on one sheet, and G, F, C, C’ and C’’ on the other. The C1-set lacks C’ and C’’, and the C2-set has a short C’, but lacks the C’’ and D strands. An additional subgroup is the IgI-set (for intermediate) that lacks C’’ and has a truncated C’ (61). The IgSF domains contain relatively few highly conserved residues, but some sequence features are always present such as the previously mentioned cysteines on the B and F strands. Additionally, the sequences that make up the $\beta$-strands usually contain alternating hydrophobic amino acids. Their side-chains point inward and make up the core of the domain.

**Figure 2.** Structure of immunoglobulin-like domains. Schematic presentation of the classical IgV set domain.
**Recognition of self**

In humans, the killer cell immunoglobulin-like receptors (KIR), encoded by the LRC, recognize MHC class I and they are the main receptors for self-recognition (62, 63). The KIR family is encoded by 12 loci, clustered within the LRC. Across the population, great differences exist in the KIR haplotypes, both in terms of gene content and allelic polymorphism. In general, the basic structure of KIR consists of two or three extracellular Ig-like domains, with either long ITIM-containing cytoplasmic domains (the inhibitory KIR) or shorter cytoplasmic domains with a charged amino acid in their transmembrane region that allows it to bind signaling adaptor molecules (activating KIR). The inhibitory KIR bind to MHC class I ligands. The activating KIR are structurally very similar to their inhibitory siblings in the ligand-binding extracellular domains, but have other ligand specificities. Ligands for activating KIR are largely unclear or yet unidentified, although some activating KIR have been reported to bind MHC class I (64). The biological reasons for pairs of activating and inhibitory NK receptors with similar MHC class I specificities seem difficult to understand and are not yet fully known. It is believed that activating KIR can detect either altered self, presented by MHC-I (65) or even MHC-independent ligands. Our lab recently discovered a β2-microglobulin independent ligand for KIR2DS2 expressed by cancer cell lines of different origin (66).

Rodents have expanded a different receptor family for MHC-I recognition (67, 68). The Ly49 receptors are expressed as disulphide-linked homodimers and belong to the C-type lectin-like receptor family encoded by the NKC. As with KIR, the inhibitory Ly49s express ITIMs in their cytoplasmic tails and bind MHC class I. Conversely, the activating Ly49s associate with activating adaptor molecules, primarily DAP12, through charged amino acids in their transmembrane regions. As with human, the identification of ligands for activating Ly49 is still in its infancy, but is has been shown that some can bind virally derived MHC-I-like proteins (Ly49H and m157, previously described). Receptors in the leukocyte immunoglobulin-like receptor (LILR) family can be expressed not only on B, T and NK cells, but also on cells of the myeloid lineage (69). Inhibitory members of this family bind both classical and non-classical MHC class I molecules, but ligands for activating LILRs remain largely unknown.

The last major family of receptors recognizing self MHC, CD94/NKG2, is present in humans and rodents as well as other mammalian species, and is encoded in the NKC. CD94 forms heterodimers with either NKG2A, -C or E (70). In most species, CD94 has no signaling function, thus intracellular signaling is mediated by the associated NKG2 molecules. NKG2A contains two ITIM and in the human binds HLA-E, a non-classical MHC class I molecule that preferentially presents signal peptides derived from other MHC class I proteins including HLA-G (71, 72). Upon association with
NKG2C or -E, the CD94/NKG2 complex becomes an activating receptor, as NKG2C and -E associate with DAP12. This is differently organized in rodents, where CD94 associates directly with the activating adaptors DAP10 and DAP12 and NKG2C and -E lack this capacity. Mouse and rat CD94/NKG2A heterodimers are thus bifunctional, harboring both ITAM and ITIM (73). CD94/NKG2A, by HLA-E recognition, provides a monitoring mechanism by NK cells to assess the expression level of class I MHC molecules, and viral infections or cellular transformations that downregulate these levels may then be detected by CD94/NKG2A\(^+\) cells. HLA-E also presents ligands other than those derived from MHC leader sequences. HCMV has been shown to utilize this, as one of its viral proteins, UL40 gives rise to a peptide identical to an endogenous peptide presented by HLA-E, driving HLA-E expression. This leads to inhibition of CD94/NKG2A\(^+\) cells (74).

**Single-family member receptors**

**NKG2D**

Despite what the name suggests, NKG2D in terms of sequence similarity does not belong to the NKG2 (KLRC) family, and does not form heterodimers with CD94 (75). Rather, it forms homodimers by disulfide linkage and binds to DAP10. In the mouse, depending on alternative splicing of the cytoplasmic tail, NKG2D can associate with either DAP10 or DAP12 (76-78). NKG2D binds ligands that are structurally similar to MHC-I, such as the polymorphic MIC-A and -B (79) or members of the RAET1 (retinoic acid early transcript 1) family, called ULBP1-4 (UL16 binding proteins) (80, 81). Rodent orthologues of NKG2D also bind MHC class I-like proteins (13, 82-85). The ligands for NKG2D are in general not expressed in healthy cells, but their expression is induced in several contexts of cellular stress, such as inflammation, DNA damage and cellular transformation and has also been reported to be a mediators of autoimmune diseases (reviewed in (86)).

**NKp44**

NKp44 was identified as an activating receptor mediating non-MHC-restricted tumor cell lysis (87). It is to some extent considered a marker of activation, as it is only expressed upon cytokine activation. NKp44 requires association with DAP12 to mediate activating signals (88). NKp44 also contains an ITIM-like sequence in its cytoplasmic tail, but there are conflicting reports as to whether this is functional or not. Several ligands have been reported to bind NKp44. These include virally derived proteins such as hemagglutinin-neuraminidases of the Sendai virus, as well as envelope glycoproteins from Flaviviruses (reviewed in (89)). Fusion proteins of NKp44 also bind to Bacillus Calmette-Guérin (BCG) and other bacterial-derived proteins (90). Among cellular ligands for NKp44, the nuclear protein proliferating cell nuclear antigen (PCNA) has been proposed (91). Another ligand called NKp44L is
partially identical to mixed-lineage leukemia protein 5 (MLLP5), and is highly expressed in several tumor cell lines (92).

NKp46
NKp46 is expressed by all NK cells and is considered the best single lineage marker molecule for NK cells in the human, rat, mouse and cattle (93-97). Discrete subsets of NKT cells and some ILC3 have also been shown to express NKp46 (93, 94, 98, 99). The receptor is encoded within the LRC and is more similar to its LRC neighbors (CD89 and the LILR family) than to other NK cell receptors. Upon ligand binding, NKp46 associates with a heterodimer of CD3ζ and FcεRIγ through a positively charged amino acid in its transmembrane region. As with NKp30 and NKp44, several ligands have been reported to bind NKp46, including viral hemagglutinins and properdin - a plasma glycoprotein known to be a positive regulator of the alternative pathway of complement activation (100), which may also be released upon infection with Neisseria meningitidis (101, 102). NKp46 has also been shown to be involved in recognition of M. tuberculosis through association with vimentin (103).

NKp30
Natural killer cell p30-related protein, or NKp30, is an IgSF receptor, also named NCR3 to indicate some functional similarities with NCR2 (NKp44) and NCR1 (NKp46). Notably, there are no significant sequence similarities between the NCRs over many other IgSF receptors, so the use of this term to indicate a genetic family is incorrect (104). NKp30 is normally expressed by virtually all human mature NK cells. Several different ligands have been proposed to engage with NKp30, to be discussed later.

NKp30 cloning, structure and splice variants
The cDNA encoding NKp30 was identified using expression library cloning of polyclonal human NK cells using a monoclonal antibody previously shown to react with an NK cell surface protein of 30 kDa (104). Independently, it was identified by RT-PCR of immune-related cell lines by EST database searches (105). NKp30 is an IgSF member, located in the extremely polymorphic telomeric end of the class III region of the MHC. NKp30 consists of a single Ig-like domain, coupled to a short stalk, a transmembrane region with a charged amino acid and a short cytoplasmic tail. The receptor lacks internal signaling motifs, but the charged arginine in the +4 position in the transmembrane region allows NKp30 to associate with aspartic acid on homodimers of CD3ζ and possibly also heterodimers of CD3ζ and FcεRIγ. These adaptors contain ITAMs that will recruit and activate Syk to initiate activation of NK cell effector functions (106). NKp30 is transcribed in six different splice variants due to alternative splicing of exons 2 and 4 (105, 107) (Fig. 3). The three versions of exon four arise either from different exon usage or alternative splicing and results in
different lengths of the cytoplasmic tail, shown to have various implications in terms of signaling and NK cell effector function. Relative frequencies of these three splice variants differ between individuals, and have been reported to affect prognosis in certain malignancies. This will be discussed in greater detail below. The splicing of the IgSF domain exon (exon 2) results in a deletion of 75 nucleotides that retains the open reading frame. This is quite unique, and to my knowledge, intra Ig-domain splicing has not been described elsewhere. Apart from the observation that it is expressed to a lesser extent that its longer counterpart, the function, expression pattern and detection of a possible ligand for this short version of NKp30 have not been greatly studied so far.

**Figure 3. The splice variants of NKp30. Exon usage and alternative splicing of exons 2 and 4 results in six different splice variants of NKp30**

NKp30 splice variants with an intact IgSF exon and differential splicing of the cytoplasmic tail are named a, b and c. These different cytoplasmic tails are functionally dissimilar (108). In experiments where the human NK cell line NKL was transfected with the different isoforms, NKp30a and NKp30b revealed an activating phenotype, as their engagement resulted in IFN-γ and TNF-α production, whereas NKp30c did not. Furthermore, NKL-NKp30c was not activated upon co-culture with immature dendritic cells or B7H6-expressing target cells, as opposed to the activating isoforms. Rather, B7H6-expressing target cells triggered the release of the inhibitory cytokine IL-10 by NKp30c transfectants. This immunomodulatory phenotype was attributed to the fact that upon crosslinking of NKp30, the -c isoform was weakly associated to CD3ζ, induced rapid phosphorylation of p38 MAP kinase, and the
downstream effects of NF-κB signaling seemed incomplete compared to isoforms -a and -b. NKp30a was the only isoform capable of triggering cytotoxicity, but both -a and -b mediated T<sub>H</sub>1 cytokine release (108).

The differential functions of isoforms a-c have been shown to have prognostic effects (108). Profile c patients (with NKp30c as the most abundant isoform in blood NK cells) showed a poorer prognosis with gastrointestinal stromal tumors (GIST) than profile ab patients. Of note, the immunosuppressive isoform c was more frequent in GIST patients compared to healthy volunteers. Similar observations were made in pediatric neuroblastoma patients (109). This suggests that differential expression of NKp30 isoforms may have prognostic value in certain malignancies. For instance, late stage melanoma patients had higher expression levels of NKp30c compared to the other isoforms (110). Long-term survivors had elevated levels of NKp30a transcript and these NK cells had increased degranulation capability, which might have played a protective role. The isoforms of NKp30 are also differentially expressed in peripheral blood compared to decidual NK cells (111). The presence of IL-15, IL-18 and TGF-β in decidual stromal tissue has been proposed to induce expression of NKp30c, whereas NKp30a/b isoforms are overrepresented in peripheral blood. Furthermore, women who experienced sporadic or recurrent miscarriages had significantly increased placental mRNA of isoforms -a and -b, suggesting that increased expression of these activating isoforms may contribute to failed pregnancies (112).

**NKp30 in NK DC crosstalk**

Both mature and immature dendritic cells (mDC and iDC, respectively) can activate resting NK cells in co-culture, resulting in IFN-γ secretion and expansion of NK cell numbers (113). This activation requires both cytokine signals and a direct NK-DC cell-cell contact (114). The synapse formation between NK cells and DCs involves cytoskeleton rearrangement, resulting in polarized secretion of IL-12 by the DC towards the NK cell (115). IL-12 will then stimulate the NK cell to secrete IFN-γ. In co-cultures with low NK/iDC ratios (1:5), activated NK cells will mediate DC activation (116), whereas high NK/iDC ratios (5:1) leads to killing of the iDC in an NKp30-dependent manner (117, 118). During inflammatory conditions, this is thought to select for a more immunogenic DC population that directs a more efficient immune response. Quite recently, NKp30 was shown to mediate killing of both iDC and T<sub>H</sub>2 polarized DCs - this was the first description of an interaction between these cell types in T<sub>H</sub>2 immunity (119).
Rat NKp30

The discovery of a novel NK cell receptor in human led to the search for orthologues in other species. All investigated laboratory strains of *Mus musculus* have an NKp30 pseudogene, where the presence of premature stop codon in exon 2 leads to generation of a severely truncated, presumably non-expressed protein. In *Mus caroli* the NKp30 gene predicts a full-length protein (120). Rat NKp30 was independently identified by two different groups (121, 122) and consists of 192 residues sharing 60.4% amino acid identity with human NKp30a. There is some degree of polymorphism between rat strains, but splice variants have not been reported in the rat. NKp30 transcripts were detected in splenic NK and T cells, and generation of monoclonal antibodies revealed its surface expression on a subset of splenic and blood NK cells (123). Rat NKp30 also induced IFNγ release, shown with transfected rat NK cell line as well as with primary NK cells.

**NKp30 on innate lymphoid cells**

Innate lymphoid cell, or ILC, is the collective name of five groups of cells derived from a common progenitor. These are separated based on transcription factor expression, surface receptor phenotype and cytokine production profile, and comprise of the following: NK cells, groups 1-3 ILCs and lymphoid tissue-inducer (LTI) cells. Functionally, ILC1, ILC2 and ILC3 can be seen as the innate counterparts of T helper 1, 2 and 17 respectively, producing cytokines that influence both innate and adaptive immune responses (reviewed in (124)). It has been shown that a large subset of ILC2s expresses NKp30, and RT-PCR revealed that the predominant isoform expressed by these cells is the immunoregulatory NKp30c (125). Crosslinking of NKp30 on ILC2s by B7H6 resulted in secretion of IL-5 and IL-13, typical type 2 cytokines, supporting a functional role for NKp30 on these cells.

**NKp30 in disease**

ILC2s have been observed to be resident in the skin of patients with atopic dermatitis, and immunohistochemistry revealed high expression of B7H6 in the suprabasal epidermis of lesional skin biopsies of patients. Of note, low levels of B7H6 were detected in basal epidermis in healthy skin. The presence of NKp30-expressing ILC2s in diseased skin where B7H6 is highly expressed could perhaps be a driver of the pathology of atopic dermatitis. Tumor-derived B7H6 has also been shown to induce IL-13 secretion by ILC2s in patients with acute promyelocytic leukemia (APL) (126). IL-13-secretion in turn promotes myeloid-derived suppressor cells (MDSCs) which will suppress antitumor immune responses. Thus, in the context of ILC2s, NKp30 can play a role as a driver of disease rather than protecting the host, either as a mediator of
autoimmunity in atopic dermatitis, or contributing to the immunosuppressive microenvironment generated by tumor cells in APL.

Based on observations in patients with hepatocellular carcinoma, it has been proposed myeloid-derived suppressor cells can suppress NK cells by a mechanism that depends on NKp30 (127). The nature of the ligand for NKp30 in this setting remains undetermined, and it is not clear whether this phenomenon is due to skewed expression towards inhibitory NKp30 splice variants.

**Ligands of NKp30**

**Galectin-3**

Galectin-3 belongs to the β-galactoside-binding lectin family, which contains carbohydrate-recognition domains that binds to β-galactosides with high affinity. Intracellular galectin-3 has been reported to promote tumor growth, survival and metastasis, whereas extracellular galectin-3 enhances tumor cell adhesion, invasiveness and even immune escape (128). In particular, it was reported that galectin-3 regulates NK cells activation and function, since enhanced activity of splenic NK cells was observed in GAL3 knockout mice. (129). Wang and colleagues found that certain tumor cell lines secreted galectin-3, which could bind to an NK cell line, and more specifically, to NKp30. Galectin-3 treatment of co-cultures of cancer cell lines with a cytotoxic NK cell line reduced expression of the degranulation marker CD107. This effect could be inhibited by pre-incubating the galectin-3 supernatant with NKp30 fusion protein. Overexpression and downregulation of galectin-3 led to reduced and increased tumor growth, respectively, in an *in vivo* xenograft mouse model (130). It has also been shown that galectin-3 blocks the interaction between B7H6 and NKp30 expressed on ILC2s (125).

**BAG-6**

The gene encoding the nuclear protein BCL2-associated athanogene 6 (BAG-6, formerly called BAT3)) is located in a cluster of immune-relevant genes in the class III region of the MHC, close to NKp30 (131). Some structural motifs have been identified, including an N-terminal ubiquitin-like domain, a long proline-rich section and a C-terminal BAG domain that can interact with heat shock protein 70. BAG-6 has been reported to be involved in several processes such as apoptosis, gene regulation, protein degradation and also in a variety of immunological pathways, such as promotion of Th1 responses and regulation of MHCII expression on APCs (132, 133). Lastly, BAG-6 has also been reported to engage NKp30 on NK cells. Pogge von Strandmann et.al. showed that endogenous BAG-6 is predominantly present in the nucleus, but can be routed from the nucleus to the vicinity of the plasma membrane. BAG-6 overexpression in 293T cells increased target cell susceptibility to NKp30-mediated lysis, whereas siRNA knock-down of BAG-6 led to reduced killing. Treating leukemia
cell lines with BAG-6 positive exosomes has been observed to induce NKp30-dependent cytotoxicity (134). There are also observations suggesting that purified BAG-6 can have an inhibitory effect on NKp30-dependent killing (135, 136). The same study also found increased level of soluble BAG-6 in the plasma of chronic lymphocytic leukemia patients compared to controls. Interestingly, exosomes isolated from these patients did not contain BAG-6. It has been reported that the C-terminal 250 residues of BAG-6 is essential for its binding to NKp30 (137). This fragment of BAG-6 could not activate NKp30 reporter cells, either in soluble form, or immobilized to mimic presentation on exosome surfaces, but was reported to block NKp30 binding to B7H6-transfected cells.

**β-glucans on Cryptococcus and Candida**

An initial study (138) suggesting that NKp30 was a pattern recognition receptor involved in recognizing and killing the fungi *Cryptococcus neoformans* and *Candida albicans* led to the search for a pathogen-associated molecular pattern on these fungi that would serve as a ligand for NKp30. β-1,3-glucan derived from *S. cerevisiae* was found to bind recombinant NKp30, and agents that block the synthesis of β-1,3-glucan reduced anticryptococcal activity by NK cells (139). Addition of β-1,3-glucan was found to stimulate the killing of *C. albicans* by an NK cell line, and to cause an increased expression and clustering of NKp30 on the cell surface.

**Duffy binding-like-1α domain of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1)**

NK cells inhibit the growth of *Plasmodium falciparum* (the parasite causing malaria) and mediated direct killing of infected red blood cells (RBC) (140). The search for the molecular mechanism to explain this led to the discovery of *PfEMP-1* as a ligand for NKp30 (141). Upon infection of RBCs, *P. falciparum* inserts parasite-derived proteins into the RBC cell membrane, creating knob-like protrusions. *PfEMP-1* consists of 2-7 Duffy binding-like (DBL) domains and is anchored in these knobs. The first of these domains, DBL1-α, forms the head of the structure and mediates adhesion. NKp30 fusion proteins bound directly to three different peptides derived from DBL1-α in an ELISA setup, and also bound to the surface of RBCs infected with different strains of *P. falciparum*. Lysis of infected target cells was blocked when NK cells were pre-incubated with DBL1-α or anti-NKp30 serum. NK cells incubated with DBL1-α showed a decrease in NKp30 expression, leading the authors to conclude that NKp30 is important in immune defense against malaria.

**pp65 - a peptide derived from human cytomegalovirus (HCMV)**

HCMV is present as an endemic latent infection in human populations. Healthy individuals usually get subclinical primary infections, followed by latent viral infection throughout the lifetime of the host, but causes disease and mortality in
immunocompromised patients. People with NK-deficiencies are highly susceptible to infections of viruses belonging to the herpes family, including HCMV. HCMV has many mechanisms of immune escape, such as expression of MHC-I-like decoy molecules and downregulation of ligands for NKG2D. Another proposed mechanism of viral escape is the interference of the viral peptide pp65 with CD3\(\zeta\), disengaging the latter from Nkp30, thereby disrupting downstream signaling and activation of the NK cell (142). Incubation of NK cells with pp65 blocked the binding of antibodies specific for Nkp30. Cells infected with a pp65 deletion mutant were more susceptible to NK cell lysis in an 18 hour killing assay, and although killing was not blocked by an Nkp30 mAb, reduced killing was observed with mouse anti-Nkp30 serum, suggesting Nkp30 dependency. Furthermore, soluble pp65 was detectable in the supernatant of infected cells and was found to inhibit Nkp30-mediated NK cell activity, as shown in a redirected killing assay. The molecular mechanism for inhibition was suggested by densitometric analysis, where a 90% decrease in association between Nkp30 and CD3\(\zeta\) was observed upon incubation with pp65.

**Hemagglutinin (HA)**

NK cells play an important protective role against Vaccinia virus (VV) and Ectromelia mousepox (ECTV) virus infection. Chisholm and colleagues showed that the increased susceptibility of target cell recognition by NK cells upon viral infection was dependent on Nkp30, Nkp44 and Nkp46 (102). HA from influenza virus was early reported as a ligand for Nkp46 (101), and later also for Nkp44 but not Nkp30 (143). Both VV and ECTV infection was shown to increase binding of Nkp30 and Nkp46 fusion proteins on cell lines, but not Nkp44 (144). Infection with HA deletion mutants, chemical reduction of HA expression or preincubation with anti-HA mAb reduced or even abrogated this recognition. Nkp46 was stimulated by binding to VV-derived HA, whereas HA interaction with Nkp30 had a blocking effect. The net result of this dual influence of NK cells was that infection with an HA deletion mutant led to increased killing of target cells, and silencing of Nkp30 by shRNA partly reduced this recognition, suggesting an Nkp30-specific inhibitory role of HA.

**Heparin and heparan sulfate proteoglycan**

Some of the most controversial ligands for Nkp30 are heparin and heparan sulfate proteoglycans. Bloushtain and colleagues found that in a co-incubation of Nkp30-Ig with different carbohydrate structures, 6-O-sulfo-N-acetyllactosamine (6-O-sulfo-LacNAc) reduced staining of Nkp30-Ig to tumor cell lines (145). 6-O-sulfo-LacNAc is a component of several carbohydrate structures including heparin. Heparin had the same inhibitory effect on Nkp30-Ig binding as observed with 6-O-sulfo-LacNAc. Treatment of tumor cells removing heparin and heparan sulfate from the surface reduced Nkp30-Ig staining, and target cells stably expressing heparanase which cleaves off heparan sulfate and heparin were less susceptible to killing. However, in a
paper published one year later, Warren et al. presented conflicting data showing that NKp30-dependent recognition and killing of heparan sulfate positive target cells by NK cells was unaffected by treatment with heparanase (146). A subsequent paper highlighted the importance of glycosylation status of the recombinant NKp30-Ig receptors used, demonstrating that altered glycosylation could affect the binding of NKp30 to heparan sulfate (147). The same group also later identified specific microdomains on heparan sulfate recognized by NKp30 (148).

**B7H6**

At the time, the absence of cell-encoded surface-bound ligands for NKp30 led Brandt and colleagues in 2009 to search for a ligand molecule that would explain NKp30-dependent recognition of K562 cells (16). Mass spectrometry of proteins cross-linked to NKp30 led to identification of B7H6 as the likely counter-structure, designated so as it showed structural similarities to the other B7 family members. Extracellularly, B7H6 consists of two Ig-like domains (distal V-set, membrane-proximal C2-set) with a short stalk region coupling it to the transmembrane domain. The cytoplasmic tail of human B7H6 contains some putative signaling motifs but the functional role is unclear. NKp30 reporter cells responded to B7H6, but notably showed no reactivity towards heparan sulfate. Soluble NKp30-Fc fusion protein could block NK cell lysis of B7H6-expressing target cells. Corroborating B7H6 as a ligand for human NKp30, the 3D structure of B7H6 bound to NKp30 has been solved by crystallography (149, 150).

**Expression and regulation of B7H6**

B7H6 is expressed by several tumor cell lines and also in samples from patients with various hematological malignancies. In contrast, albeit with some exceptions (125, 151), little evidence supports surface expression of B7H6 on normal, healthy cells, to the extent that this has been investigated in all tissues. Unstimulated PBMCs do not express surface B7H6, but upon 48 hour microbial or inflammatory stimulation in vitro, surface expression of B7H6 on monocytes and neutrophils was detected. The increased expression was sufficient to activate NKp30 reporter cells. In 65% of sepsis patients, proinflammatory monocytes had increased surface expression of B7H6, whereas healthy donors were negative. Furthermore, soluble B7H6 was detected in serum from sepsis patients, restricted to those with a gram-negative infection (152).

Investigating immunoregulatory roles of different HCMV genes, Fielding and colleagues discovered that cells infected with a deletion variant of HCMV expressed relatively high levels of B7H6 compared to the wild-type virus (153). This suggested that HCMV has developed specific tools to suppress B7H6 expression to evade NK cell killing, again suggesting that B7H6 expression could be expressed on infected cells, marking them out for killing by NK cells. In particular, the HCMV genes US18 and US20 were found to downregulate the expression of B7H6 on infected cells, leading to reduced NK cell killing of the fibroblasts. Another group has reported data
corroborating B7H6 regulation by HCMV (154). The two groups used complimentary approaches with different target cells and different HCMV strains (Merlin and TB40-E), suggesting that B7H6 downregulation could be a universal mechanism of HCMV immune escape.

Several reports have studied to what extent B7H6 is associated with various cancers. In non-small cell lung cancer, high expression was observed in 10% of patients, but no correlation was found between B7H6 expression and disease pathology (155). Increased B7H6 expression was also detected in ovarian cancer and glioma tissues, and its expression correlated with metastasis status (156, 157). Furthermore, in more than 50% of patients with seropapillary ovarian carcinoma, a reduced NKp30 expression was detected in tumor-associated NK cells from peritoneal fluids compared to peripheral blood NK cells (158). This reduction in expression was attributed chronic engagement of NKp30 by B7H6, either in soluble form or expressed by tumor cells. Elevated levels of soluble B7H6 were also detected in sera from pediatric patients with neuroblastoma (109, 159). In glioma as well as hepatocellular carcinoma cell lines, B7H6 knock-down decreased proliferation, migration and invasion in in vitro assays (160). Thus, so far, the literature does at large support the classification of B7H6 as a tumor-specific marker.

Despite the increasing knowledge of where B7H6 is expressed, the molecular mechanisms that regulate its expression are largely unknown. It has been shown that inhibition of histone deacetylase inhibitors (HDACs) can downregulate B7H6 surface expression on tumor cells, leading to reduced NKp30-dependent effector function by NK cells (161). HDACs are enzymes that remove acetyl groups from various proteins, resulting in modulation of gene expression. Another study identified a binding motif for the proto-oncogene Myc in the promoter region of B7H6, and siRNA/shRNA knockdown of Myc weakly reduced B7H6 expression in tumor cells (162), but wider investigation is needed to understand why cancer cells express B7H6.

The expression of soluble B7H6 has been observed in several diseases, and shedding of B7H6 may represent an important strategy used by cancer cells to escape NK cell recognition. In some cancer cell lines this is mediated by metalloproteinase activities of ADAM-10 and -17 (28). siRNA knockdown or pharmacological inhibition of ADAM10/17 rescued surface expression of B7H6 and restored NKp30-mediated activation of NK cells. As these metalloproteases are ubiquitously expressed in a wide range of cancers, they remain attractive targets in anti-cancer therapy. Soluble variants of B7H6 have also recently been reported in pregnancy (163). Using western blotting and mass spectrometry, two distinct soluble B7H6 variants (30 and 37kDa) were detected in the serum of pregnant women (in both the exosome and exosome-free fraction), and were maintained throughout the entirety of the pregnancy. How B7H6 is released in pregnancy remains to be determined and independently confirmed.
**B7H6 in cancer therapy strategies**

Several treatment strategies could be devised to target B7H6, relying on the observations that this molecule appears not to be expressed in healthy tissues. Wu and colleagues have shown how both chimeric antigen receptors (CARs) and bispecific T cell engagers (BiTEs) target B7H6-positive tumors in vitro as well as in vivo (mice), promoting T cell-mediated antitumor actions (164-166). The same lab recently also successfully humanized the B7H6-specific CAR to eliminate the possibility of host-anti-CAR responses (167). Inversely, by using B7H6 as a tool to trigger NKp30 on NK cells, a B7H6-CD20 bifunctional fusion protein has been used towards lymphoma. This protein consists of a single chain variable fragment (scFv) towards CD20 that will target B cells, and the ectodomain of B7H6 that will engage NKp30, resulting in NKp30-mediated NK cell responses towards the CD20⁺ lymphoma cells cells (168, 169). Similarly, the ectodomain of B7H6 was fused to the scFv of an antibody towards human epidermal growth factor receptor 2, a protein known to be expressed by many solid tumors leading to enhanced NKp30-mediated antitumor responses (170).

The various ligands for NKp30 are summarized in Table 1.

**Table 1. Ligands of NKp30**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>NKp30 engagement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA of the ectromelia and vaccinia virus</td>
<td>Inhibition</td>
<td>(144)</td>
</tr>
<tr>
<td>Released pp65 of HCMV</td>
<td>Inhibition</td>
<td>(142)</td>
</tr>
<tr>
<td><em>PfEMP1</em> of <em>Plasmodium falciparum</em></td>
<td>Activation</td>
<td>(141)</td>
</tr>
<tr>
<td>Heparin and heparan sulphates</td>
<td>Activation</td>
<td>(145-148)</td>
</tr>
<tr>
<td>BAG-6</td>
<td>Activation/Inhibition</td>
<td>(134)</td>
</tr>
<tr>
<td>B7H6</td>
<td>Activation</td>
<td>(16)</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>Inhibition</td>
<td>(130)</td>
</tr>
<tr>
<td>β-glucans of fungi</td>
<td>Activation</td>
<td>(139)</td>
</tr>
</tbody>
</table>

**The CD28 receptor family and the B7 family of ligands**

NKp30 and B7H6 belong to the CD28 and B7 families respectively. These two groups of proteins are primarily known for their role in T cell activation and maintenance of peripheral tolerance and consist of a large - and continuously growing - group of receptors and ligands (Fig. 4). The concept of a two-signal model of lymphocyte activation was first proposed by Lafferty (171). The model suggested that in order for a naïve T cell to become activated, it needs both the antigen specific signal provided through peptide-bound MHC and the TCR, but in addition there is a need for a secondary stimuli, now known to be mediated by T cell expressed CD28 in interaction with B7-1 or B7-2 on the APCs. The latter interaction is referred to as signal two, and in its absence, the T cell fails to activate properly and becomes anergic. The model is
somewhat simplified, as it has been shown that T cells may become activated in the absence of signal two, provided they receive a strong TCR signal (172-174). CD28 is constitutively expressed and upon engagement with its ligands, it promotes survival and cytokine production that in turn will initiate clonal expansion and differentiation (175-178). In the absence of TCR signaling, CD28 engagement rarely has any physiological effects.

Not only positive signals are delivered to the T cells. In order to prevent an overreactive immune response that might lead to tissue damage, autoimmunity etc., T cell activation is terminated by other members of the CD28 family. The best characterized member is cytotoxic T-lymphocyte associated protein 4 (CTLA-4). Both CD28 and CTLA-4 have dual specificity for B7-1 and B7-2 but CTLA-4 is the higher-affinity receptor for these two ligands. It is rapidly upregulated following T cell engagement, and primarily by competition for ligand, will inhibit CD28 signaling and T cell activation. The importance of this termination function was demonstrated in Ctl4−/− mice, which became fatally ill due to overproliferative lymphocytes (179-181).

**Figure 4.** The CD28 and B7 family members. CD28 members are predominantly expressed on T cells, some are expressed elsewhere such as TMIGD2 on endothelial cells (EC) and NKp30 on NK cells. The B7 ligands are usually expressed on professional antigen presenting cells (APC) or tumor cells.
Inducible co-stimulator (ICOS) and its ligand (ICOS-L) represent another pair of molecules that, as their name suggests, provides co-stimulatory signals to T cells (182-184). ICOS is weakly expressed on naïve T cells, but is rapidly upregulated following TCR and CD28 engagement. By binding to its ligand, ICOS promotes cell proliferation, survival and differentiation, and increases secretion of several cytokines that in turn stimulate T_{H1}, T_{H2} or T_{H17} function. Of note, unlike CD28, ICOS does not induce IL-2 secretion, thus its contribution to T cell activation is considered somewhat more moderate. ICOS-L expression by B cells, monocytes, DCs and T cells is induced by inflammatory mediators such as TNFα. It can also be expressed by non-haematopoetic cells such as endothelial cells, thereby playing an important role in reactivation of T cells in the endothelium as well as homing of immune cells to inflamed tissue (185). ICOS plays an important role in the formation of germinal centers and in antibody class switching, by differentiating T cells into T follicular helper cells (186). Furthermore, ICOS-ICOS-L interaction induces reverse signaling through ICOS-L, enhancing the immunogenic function of dendritic cells by increasing their secretion of IL-6 (187). In terms of negative immune regulation, it has been shown that the IL-10 and TGFβ secretion following ICOS engagement regulates the function of T_{regs}, and tumor-infiltrating ICOS^+ T_{regs} have a stronger suppressive ability than their ICOS^- counterparts (188, 189).

Programmed death 1 (PD-1) was first identified in a T cell hybridoma undergoing cell death, and was hence given its name, but it was later understood that this receptor is upregulated upon cellular activation (190-193). PD-1 is expressed by activated T cells, B cells and monocytes as well as γδ T cells and activated NK cells (194). The two ligands of PD-1 have diverse expression patterns. PD-L1 can be expressed on T, B cells, macrophages, DCs, bone marrow-derived mast cells as well as various non-haematopoetic cells (195-197). PD-L2 on the other hand, shows a more restricted expression pattern, being expressed by DCs, macrophages and bone marrow-derived mast cells (198, 199). PD-1 binding to its ligands induces signaling cascades that interfere with signals mediated by activating receptors, resulting in a dampening of the immune response. The importance of PD-1 in maintaining peripheral tolerance was shown in Pd1^-/- mice, where the few autoreactive T cells that escape thymic selection caused a lethal graft-versus-host-like disease compared to wildtype mice (200).

Other members of the B7 family exist, but their receptor counterparts have yet to be identified. These include B7H3, B7x, B7-H5 and HHLA2 and have been shown to have both immunostimulatory as well as immunomodulatory effects (reviewed in (201)). B7H3 (CD276) binds an unknown receptor on activated T cells, and has also been found to bind a receptor on monocytes and peritoneal macrophages from septic patients (202, 203). In an immune response, B7-H3 may exhibit both an activating and inhibitory role as has been shown in conflicting reports. On one hand, B7-H3 has been
shown to induce proliferation of T cells (202), but others have shown the opposite (204, 205). This dual activity of B7-H3 could be explained by association to different binding partners, but this remains to be determined. B7x (B7-H4, B7-S1 or Vtcn1) expression is restricted to APCs after in vitro stimulation. Engagement with its still unknown receptor on T cells results in downregulation of T cell proliferation and IL-2 production (206, 207). Another co-inhibitory molecule is B7-H5 (VISTA, GI24, Dies1, PD-1H). It is expressed highly on myeloid cells, and to a low extent on T cells, but not NK or B cells (208). APC expression of B7-H5 inhibits T cell proliferation and cytokine production (209), and its high expression in human placenta may play a role in fetal tolerance. HHLA2 (B7-H7) has been shown to engage TMIGD2 on endothelial cells where it might promote angiogenesis (210, 211). HHLA2 has also been shown to bind TMIGD2 on T cells. Whereas one report observed co-stimulation of proliferation (212), others have shown an inhibitory function of B7-H7 on T cells (213). As with B7-H3, these contradicting reports could be explained by several binding partners.

**CD28-B7 interactions in cancer immunotherapy**

The interactions of the CD28 and B7 families play major roles in immune regulation, including in tumor immunity. A large number of publications report the increased expression of inhibitory B7 ligands by tumors, driven by the high selective pressure exerted on the tumor cell to escape attacks by immune cells. Inflammatory cytokines in the tumor microenvironment and aberrant gene expression are thought to be the main drivers of increased expression (reviewed in (214)). In general, a high degree of inhibition is associated with poor prognosis. Furthermore, shedding of ligands for activating CD28 family members by cancer cells has also been reported (e.g. B7H6), resulting in blockade of the receptor, rendering the immune cell unable to attack the target. Manipulating these pathways to enhance co-stimulation and inhibit suppression has resulted in the growing field of research to promote anti-tumor immunity. In mice, some success has been observed with transplantable tumors, where B7-1 was induced by transfection resulting in increased tumor recognition by T cells and rejection of the tumor (215-217). This also provided the mice with tumor-specific memory T cells able to reject the tumor cells upon re-challenge. A similar approach has shown moderate success in human clinical trials, where either autologous tumor cells or tumor cell lines were transfected with B7-1 and injected into the patient, together with systemic IL-2 administration (218, 219). However, it is evident that this approach is not sufficient to eradicate the tumor.

Another tactic to increase co-stimulation of T cell immunity was the so-called superagonist for CD28, TGN1412. The rationale behind the trial was to increase activation through CD28, bypassing B7-1 and B7-2 interaction, leaving CD28 constitutively activated. Despite promising animal studies, the result was almost fatal to the participants involved, as they all experienced severe adverse effects due to
cytokine storm as a result of overactive T cells (220). This has led the field away from increasing stimulation and towards blocking inhibitory co-receptors, known as checkpoint blockade or checkpoint inhibition.

As several cancers upregulate their inhibitory B7 ligands to dampen anti-tumor T cell responses, the inhibition exerted by the tumor is a major hurdle to overcome in tumor specific immunity. By blocking the inhibitory receptors or their ligands, the T cell response can be restored. Ipilimumab is a monoclonal antibody that binds CTLA-4, thus preventing it from occupying B7-1 and B7-2, and allowing these to engage CD28 instead (221-223). This has proved a great success in several cancers, including melanoma, renal carcinoma and non-Hodgkin’s lymphoma. PD-1/PD-1 ligand blockade has shown very good results with melanoma, non-small cell lung cancer and several other forms of cancer (224, 225). Several antibodies have been approved by the FDA and are implemented in treatment. Other B7 members may also be efficient targets, such as B7-H3 and B7x. In addition to inhibiting T cell responses, they are both shown to be expressed in tumor vasculature, which could be a useful target to suppress tumor growth. The remarkable therapeutic success of suppressing inhibition via CD28/B7 family interactions has inspired hope that new successful immunotherapies along similar lines will follow in the near future. As expected a priori, autoimmune disease is a major observed side effect of checkpoint inhibition therapy. This is mostly due to reduced peripheral tolerance resulting from lack of inhibition of autoreactive T cells, but also because B7 family expression is not restricted to tumor cells.

Activated NK cells can express PD-1, CD28 and CTLA-4. NK cells are frequently present in tumors. The relative contribution of NK cells in checkpoint inhibition therapies remains to be investigated.
<table>
<thead>
<tr>
<th></th>
<th>NKp30</th>
<th>CD28</th>
<th>CTLA-4</th>
<th>ICOS</th>
<th>PD1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expression</strong></td>
<td>NK cells, ILC2</td>
<td>T and NK cells</td>
<td>T, B and NK cells</td>
<td>T cells (not naïve)</td>
<td>T, B, NK and myeloid cells (after activation)</td>
</tr>
<tr>
<td><strong>Cellular ligands</strong></td>
<td>B7H6, BAG6, Gal-3</td>
<td>B7-1, B7-2</td>
<td>B7-1, B7-2</td>
<td>ICOS-L</td>
<td>PD-L1, PD-L2</td>
</tr>
<tr>
<td><strong>Main function</strong></td>
<td>Activates NK cells</td>
<td>T cell co-activation</td>
<td>Prevents effector cytokine production and T cell proliferation</td>
<td>Activate T cells, Th differentiation, Ab class switch</td>
<td>Prevents effector cytokine production and cell cycle progression</td>
</tr>
</tbody>
</table>
METHODOLOGICAL CONSIDERATIONS

Cell culture
Most of the experiments performed in this thesis relied on the in vitro culture of cells. It is important to provide a reproducible environment where experiments can be repeated with the same prerequisites. Parameters such as the cell confluence, pH of cell medium and latent infection (e.g. mycoplasma) may greatly affect the phenotype of the cells and hence also result in experimental variation. In all our experiments we aimed at keeping these conditions constant, carefully considering feeding and confluence before experiments and routinely screening for mycoplasma infection by PCR.

Cell lines
Cell lines have been permanently adapted to culture, being able to divide indefinitely provided addition of nutrients, sufficient space and correct temperature, humidity and CO₂ concentration. They are typically derived from tumors or from primary cells immortalized by viral transformation. Cell lines are easily obtained from different suppliers and easy to handle compared to primary cells as they can be cultured for longer periods of time and are mostly robust with regards to culture conditions. The major disadvantage of cell lines is that they are less physiologically representative than primary cells, often genetically modified or epigenetically reprogrammed. To rule out contamination with other cell lines, most of the cells used in this study were genotyped to confirm their identity.

Cellular reporter assays
There are several ways to study receptor-ligand interactions. In this thesis, we wanted to approach physiological conditions that would mimic in vivo conditions. The physiological relevance of a receptor-ligand interaction is confirmed by an affinity sufficient to induce intracellular signaling upon cell-cell contact. To this end, BWN3G, a mouse T cell line (BW5147) stably transfected with EGFP under the regulation of a promoter containing three NFAT elements, was used (226). BWN3G cells were transfected with chimeric constructs consisting of the extracellular domain of NKp30 or B7H6 fused to the transmembrane region of human CD8 and the cytoplasmic tail of mouse CD3ζ. Upon binding to this chimeric receptor, the ITAMs in CD3ζ become phosphorylated initiating an intracellular signaling cascade resulting in the recruitment of NFAT to the promoter region of EGFP resulting in EGFP production that can be measured by flow cytometry. This assay will closely mimic the in vivo situation as the cells require the formation of immunological synapses where proteins cluster, thereby integrating avidity changes. Disadvantages of using cellular reporters can mostly be handled using proper controls. Unspecific activation of the reporter cells has been observed, but these false positive results can be controlled for by including
untransfected BWN3G and also reporters with irrelevant receptors. Furthermore, although the system is sensitive, as the reporters are able to respond to low levels of ligand on the target cell, we cannot exclude that it fails to detect ligand positive target cells if unidentified co-receptors are required.

**Transfections**

Transient transfections were performed in CHO or 293T cells. Polyethyleneimine (PEI) is a positively charged lipid polymer that will bind the negatively charged DNA of the expression constructs, allowing the DNA to bind to cell membranes and be taken up by the cell. PEI transfection of CHO and 293T resulted in transfection efficiencies up to 95% while maintaining at least 80% viability (trypan blue).

Stable transfections were performed using electroporation. High voltage electrical pulses temporarily disrupt the membrane integrity of cells by forming small pores, allowing the cells to take up plasmid DNA. Two different wave forms can be used: Square wave pulse rapidly increases to a set voltage level which it maintains throughout the duration of the pulse length, before it quickly turns off, and the process is repeated. Exponential decay, on the other hand, rapidly increases to a set peak voltage before it declines over time. We have used the latter in all our stable transfection experiments. Certain parameters are important to pay attention to, in order to achieve minimal cell death yet high transfection efficiency. Apart from pulse length and voltage which have been carefully optimized, the cells are kept at 4°C both prior to and directly after electroporation. This will prevent overheating of the sample, which may result in increased cell death, but will also allow the pores to stay open longer thereby increasing uptake of DNA. In our hands, post-transfection cell death rates were approximately 40-50%.

For transient knockdown of B7H6 expression in human tumor cell lines we used small interfering RNA (siRNA). Once inside the nucleus, the short double stranded RNA will bind the complimentary target mRNA sequence and mark it for degradation. There are several ways to deliver siRNA to a cell including cationic lipid- or polymer-based transfections, electroporation or even virus-mediated delivery. To optimize transfection efficiency, we tested various concentrations of siRNA, cell confluency, duration of transfection and also different cationic lipid formulations. Upon comparing lipofectamine RNAiMAX, PEI, Lipofectamine and Lipofectamine 2000, the two former performed better than the other two. Furthermore, RNAiMAX showed better efficiency at lower siRNA concentrations which is beneficial as it may reduce off-target effects. Transfection efficiency peaked 72-96 hours post infection. Control siRNA was always included in our experiments, to control for the general effects of transfection and introduction of foreign RNA.
Methyl transferase inhibition

DNA methylation is an epigenetic phenomenon where DNA methyl transferases (DNMT) transfer a methyl group from S-adenosyl methionine to the fifth carbon on cytosines in CpG dinucleotides. CpG motifs are infrequent in the mammalian genome, but tend to cluster (CpG islands) in the vicinity of the transcription start of genes, i.e. in the promoter region. Methylation of the promoter region will lead to repressed gene expression due to reduced accessibility for transcription factors. To study whether DNA methylation was causing the lack of B7H6 expression by tumor cell lines, we treated the cells with methyl transferase inhibitors (227). 5-aza-2’-deoxycytidine is one such compound, which is a deoxyribonucleoside analogue that is directly incorporated into the DNA. There it will “trap” the DNMT by preventing resolution of a covalent reaction intermediate, resulting in degradation of the enzyme. Another strategy is the use of small molecules that will block the catalytic pocket of the methyl transferase, thereby preventing methylation. One such example is RG108 which is the first rationally designed DNMT inhibitor. The major caveat of using these agents is the lack of gene-specific effects and the following toxicity it elicits on the cells. Treatment with DNMT inhibitors will affect the methylation status of the whole genome, inducing expression of many proteins not normally expressed. Thus the phenotype and morphology of the cells are visibly altered after treatment.

Image Stream analysis

In both paper I and II we used imaging flow cytometry (IFC)/ImageStream to study subcellular location of B7H6. Imaging flow cytometry combines features of standard flow cytometry and conventional fluorescent microscopy in one platform. The ImageStream system detects both fluorescent signals from single cells in addition to acquiring fluorescent images of the individual cells. The capacity to analyze tens of thousands of cells provides this system with a great advantage in terms of statistical analysis compared to classical microscopy of a handful of cells. In both experiments where the ImageStream technology was used 10,000-20,000 cells were acquired per sample. Also, only one fluorescent dye was analyzed so there was no need for compensation. Viable, singlet cells were gated based on aspect ratio and area, and then, based on gradient RMS (root means square) value, only data from cells in focus was acquired. The gating strategy is shown in Figure 5. To measure the signal from the fluorescent dye, several parameters were used.

In Paper I we wanted to determine whether the unusually long signal peptide of rat B7H6 was functional as an ER sorting signal, and CHO-K1 cells were stably transfected with full length rat B7H6 coupled to an intracellular YFP tag. To study the localization of rat B7H6 the Intensity feature was applied. This measures the strength of the fluorescent signal. The cells were stably transfected and originated from a clone already tested by conventional flow cytometry, known to have high expression (and
thus transfection efficiency). Therefore, of most interest in this setting was the localization of the protein and whether it was expressed at the surface or not, which was confirmed by the images acquired.

In paper II we discovered that several tumor cell lines had high levels of B7H6 RNA but no protein expression on their surface. By staining for both extracellular and intracellular B7H6, we were able to visualize the cellular distribution of the protein, and determine whether the RNA present in B7H6 surface negative cells was translated but somehow retained. In order to compare cytosolic and membrane expression, distinct masks were applied. A mask fractionates the cell into sub-compartments of choice, in this case membrane and cytosol, and allows analysis of a given feature in that compartment only. Then we could analyze the cytosolic and membrane expression separately compared to isotype control. We used three different parameters to measure the fluorescent signal; Intensity, Bright Detail Intensity (BDI) 3 and BDI7. BDI is a feature that, rather than computing all intensity detected in the cell, it only computes intensity of bright spots of 3 or 7 pixels or less in size. This excludes local background around the spots. The results obtained were similar, independently of which intensity feature we chose.

The major limitation of imaging flow cytometry compared to conventional confocal microscopy is the low resolution. This may pose a problem when studying co-localization of proteins, as two fluorescent dyes in close proximity may be perceived as co-localized even if that’s not the case. However, for our purposes, the technique is readily available and easy-to-use and provides a powerful tool to analyze cellular distribution of a single protein.

**Figure 5.** Gating strategy in ImageStream analysis. Left panel: Viable, singlet cells were gated based on area and aspect ratio. Middle panel: Gradient RMS allows for acquisition of data only from cells in focus (gate R3). Right panel: Representative image of a cell population with high expression of the marker in question.
AIMS OF THE THESIS

NKp30 is an important activating receptor, expressed by most activated NK cells. NKp30 has been reported to bind several highly different ligands. While it is quite feasible for a receptor to have more than one binding partner, the field has remained somewhat unsettled with regards to the ligand specificity of NKp30.

Therefore, the first aim of the thesis was to investigate whether the cellular expressed ligand B7H6 and its interaction with NKp30 is conserved in other mammalian species.

B7H6 expression is reported to be almost exclusively expressed on cancer cells in human. Increased knowledge of the mechanisms that induce B7H6 expression in tumor cells, while downregulating it in healthy cells, could have important implications for anti-cancer therapy.

The second aim of the thesis was to gain in-depth knowledge of the different mechanisms that regulate the expression level of B7H6 in tumor cell lines.

Lastly, cytoplasmic region splice variants of NKp30 are associated with different prognosis in cancer. To date, no functional studies have been performed to investigate the intriguing phenomenon of intra-exonic splicing of the extracellular Ig-like domain.

The third aim of the thesis was to investigate the functional role of this shorter splice variant, including regulation of splicing and expression levels in different cell types.
SUMMARY OF RESULTS
This section summarizes the experimental data and conclusions in paper I-III.

**Paper I. B7H6 is a functional ligand for NKp30 in rat and cattle and determines NKp30 reactivity toward human cancer cell lines.**

Several ligands have been suggested to bind the activating NK cell receptor NKp30, and the field is somewhat unsettled. One of the counter structures described to bind NKp30 is the B7 family member B7H6. In order to see whether the interaction between NKp30 and B7H6 is conserved through evolution, we identified full length orthologues in rat and cattle. Sequence analysis comparing B7H6 in human, rat and cattle revealed marked differences, in particular in the exons encoding the signal peptide as well as stalk, transmembrane region and cytoplasmic domains. Despite this, phylogenetic analysis comparing members of the CD28 and B7 families in human, rat and cattle, B7H6 clusters together.

In cell-cell contact-dependent assays with cells expressing chimeric NKp30 reporter constructs, interaction with B7H6 positive targets was observed in both species. Similarly, reporter cells expressing rat B7H6 responded strongly towards NKp30 expressing target cells. This demonstrates that B7H6 and NKp30 are a functional ligand-receptor pair in rat and cattle. In order to determine whether expression of B7H6 resemble the tumor-restricted pattern observed in human, we performed RT-PCR and qPCR on cDNA derived from rat tissues and cell lines. RT-PCR revealed high expression in several tissues including testis, muscle and spleen, and qPCR analysis showed high expression levels on cells of the myeloid lineage. No rat mAb exists for B7H6, so we could not determine whether RNA is also translated to protein. However, our rat NKp30 reporter cells were strongly activated towards the B7H6 positive cell lines, indicating that they do express B7H6 as a cell surface protein.

Lastly, we investigated a large panel of human tumor cell lines for their expression of B7H6 as well as their ability to activate human NKp30 reporter cells. B7H6 was expressed in a continuum on the cancer cell lines, and this variety in expression was also reflected by the tumor cell lines ability to activate the reporter cells. Furthermore, siRNA knockdown of B7H6 in tumor cell lines also reduced NKp30-dependent recognition, supporting a highly correlative relationship between B7H6 expression and NKp30 recognition.
Paper II. Epigenetic and post-transcriptional regulation of B7H6 surface expression by cancer cells

The mechanisms that regulate the expression of B7H6 are largely undefined. It has been shown that the proto-oncogene Myc can weakly enhance expression, and there is also some evidence for the involvement of histone deacetylases. In Paper II we used carcinoma cell lines with different expression levels of B7H6 to gain increased knowledge explaining why some are able to repress B7H6 surface expression. By performing bisulfite conversion we studied the methylation profile of the CpG rich stretch of the B7H6 promotor region. The first 150 bp did not seem important for regulation of transcription, as this region was methylated even in B7H6\textsuperscript{bright} cell lines. The remaining downstream region was completely unmethylated in B7H6 expressing cells, and completely methylated in two B7H6\textsuperscript{neg} cell lines. In the negative cell lines, chemical inhibition of methylation could partially induce B7H6 expression and recognition by NKp30 reporter cells. Importantly, the three B7H6\textsuperscript{low/neg} cell lines MCF7, U87 and DU145 had unmethylated promoter regions indicating that these cell lines downregulate B7H6 expression by other mechanisms.

We next performed qPCR analysis to assess the RNA level of B7H6, and surprisingly found that U87 and DU145 had very high levels of RNA despite their lack of surface expression. Using imaging flow cytometry we could not detect any intracellular retention of B7H6 protein. The presence of allelic variation or mutations could lead to rapid degradation due to improper folding, to deficient splicing, or change the reactivity to the antibodies used. Sequencing of full open reading frame of U87 RNA showed no such mutations when compared to a reference sequence. DU145 cells on the other hand exhibited a missense mutation leading to exchange of a threonine with an isoleucine in the Ig-like domain. This could be a cancer-specific mutation. Although additional experiments are necessary, these findings suggested that cancer cells can suppress B7H6 surface expression by post-translational mechanisms leading to protein degradation.
Paper III. Intra-exonic splicing of the immunoglobulin domain of NKp30

The activating NK cell receptor NKp30 is expressed as six different splice variants; in addition to differential exon usage or alternative splicing that gives rise to three cytoplasmic tails, the extracellular Ig-like domain contains splice signals that removes 75 nucleotides between the C and E strands. This splicing retains an open reading frame. Intra-exonic splicing in an IgSF domain has to our knowledge not been described for other IgSF receptors. We performed sequence alignments of NKp30 from several species and found that the features necessary for splicing (e.g. donor and acceptor sites, as well as polypyrimidine tract and a branching point adenosine), were intact in many of the mammalian species investigated. We also performed first strand synthesis at high, medium and low temperatures to rule out the possibility that the splicing was merely a cDNA synthesis artefact due to secondary structure formation.

In order to see whether there was a correlation between sequences in the cytoplasmic region and Ig domain splicing, we transfected an NK cell line with full length NKp30 constructs encoding the different cytoplasmic tails. The NKp30-S short splice variant was detected irrespective of which cytoplasmic tail was expressed. Notably, 293T cells were not able to perform splicing of the NKp30 Ig domain, suggesting that the intra-exonic splicing of the NKp30 Ig domain is a cell-specific mechanism. Lastly, NKp30-S was readily expressed at the cell surface in transfected cells, suggesting that is passed the folding control of the ER. It was not, however, recognized by a widely used anti-NKp30 mAb and did not recognize the NKp30 ligand B7H6 in cellular reporter assays.
DISCUSSION

The objective of this section is not to interpret or validate experimental data and methods presented in the articles, as that has already been thoroughly described in the manuscripts and previous sections. Rather, I will focus on discussing our findings in a broader context.

The numerous ligands for NKp30

No less than eight different ligands have been proposed to bind NKp30. Several are pathogen-encoded, such as parasitic *PfEMP-1*, β-glucans of fungi and viral HA and pp65, and then there are the cellular expressed ligands heparin/heparan sulfates, galectin-3, BAG-6 and B7H6. Although not theoretically impossible, it does seem unlikely that a single receptor is capable of recognizing such a diverse range of completely different structures of varying origins. Even though binding is observed in an experimental setting, it does not necessarily validate the interaction as functionally relevant in an *in vivo* setting.

We cloned rat and bovine B7H6 and confirmed that the interaction with NKp30 is conserved across species separated by 100 million years of evolution. This supports the functional relevance of this receptor/ligand pair. Furthermore, the fact that high correlation between B7H6 expression and NKp30 response was observed in cell-cell contact-dependent assays, and this correlation was maintained in B7H6 siRNA knockdown experiments, indicates that, at least in the cancer cell lines tested, B7H6 is the only physiologically relevant ligand for NKp30. The obvious question is whether any of these cell lines express the other reported ligands such as galectin-3 or BAG-6. From the available literature, galectin-3 has been reported to be expressed by several of the cancer cell lines in our studies at the mRNA and protein levels, so it could in theory be secreted from the tumor cell lines and block NKp30 (228-232). To our knowledge, the nuclear protein BAG-6 is ubiquitously expressed, and is in some conditions released from cells in exosomes. BAG-6 is expressed in the cell line MCF7, but is only released upon overexpression of nSMase-2 - a protein important for exosomal release (136).

BAG-6 is, apart from B7H6, the most widely studied ligand for NKp30. It has been shown to play a dual role upon binding to NKp30, depending on whether it is in purified soluble form, or immobilized by attachment to exosomes. In the former setting, soluble BAG-6 has been demonstrated to mediate inhibitory functions, such as prevention of cytokine release and NKp30-dependent killing. The C-terminal fragment of BAG-6 reported to be important for binding to NKp30 was able to block binding to B7H6. Soluble BAG-6 has been detected in serum of cancer patients suggesting a mechanism of tumor escape from NK cells. However, when expressed on exosomes, BAG-6 induced the opposite effect by activating NK cell responses. This was
demonstrated by increased cytokine release by NK cells in response to BAG-6 positive exosomes. However, when plate-bound to mimic the exosomal situation, BAG-6 did not cross-link the receptor, thereby contradicting previous findings (134, 137). This should inspire further investigation into the physiological relevance of BAG-6 as an activating ligand for NKp30. Lastly, NK cells were found to recognize and kill immature dendritic cells (iDCs) in an NKp30-dependent manner (117). This recognition was later attributed to BAG-6 (135, 233). Notably, RT-PCR detected expression of BAG-6 in iDCs, but no surface expression of the ligand has been demonstrated (134). Exosomes released from iDCs show little, if any, expression of BAG-6 (135), thus the role of BAG-6 in NK-DC crosstalk requires further investigation. Also, the molecular mechanisms for how BAG-6, being a nuclear protein with no predicted transmembrane region that will allow anchoring in plasma or exosome membranes interacts with a surface receptor, remains somewhat elusive.

An interesting hypothesis that may partly explain the various ligand specificities for NKp30 is alternative splicing of exon two of the receptor. We have shown that the splicing of full length NKp30 into the NKp30-S version seems to be cell-specific, as it was not observed in non-NK cell transfected systems. This may represent a potent mechanism to regulate NKp30 effector function, as the shorter version did not bind B7H6 in our experiments. However, it is intriguing to think that NKp30-S might engage one or several of the other ligands proposed for NKp30. In NK cell lines, and also in peripheral blood NK cells, NKp30-S seems to be constitutively expressed, although at a lower level than its full length counterpart. Still, the mechanisms regulating its expression remain unknown. The fact that the internal Ig-domain splicing signals are conserved in a majority of placental mammalian species investigated, suggests that NKp30-S has some functional importance.

Another structural feature of NKp30 not discussed in any of the papers in this thesis, is the extracellular hydrophobic, glycine-rich stretch in the stalk region, just outside the transmembrane domain. Sequence analysis showed that this sequence motif is highly conserved across species (Fig. 6A). Based on crystal structures of NKp30, the hydrophobic amino acid side chains seem to be unusually exposed to the solvent (Fig. 6B). We thus hypothesize that this hydrophobic/glycine-rich area may take part in either binding to an alternative ligand or in dimerization of receptors. Although this was not observed in the NKp30 crystals, this area could help form homodimers or, perhaps more controversially, heterodimers with other receptors. By dimerizing with another receptor, NKp30 could perhaps gain a different signaling function. Heterodimerization could also provide NKp30 with additional ligand specificities, which again could explain why so many ligands have been reported. A recent report proposes NKp30 oligomerization (234).
The expression pattern of B7H6 is largely restricted to tumor cells, corroborated by numerous studies of various cancers as well as the absence of B7H6 transcripts in healthy tissues described in previous sections (109, 155-158, 160, 170, 235, 236). Soluble B7H6 is also detected in serum of many cancer patients, and it is thought to be a mechanism of tumor escape. Of note, we harvested supernatant from several B7H6+ cell lines and tested whether they would activate our NKp30 reporter cells. No activity was seen, suggesting that B7H6 shedding is not a universal mechanism of tumor cells. However, soluble B7H6 should be investigated as a diagnostic marker. The propensity of B7H6 to be expressed by cancer cells makes it a potent target for anti-cancer therapies. Knockdown of B7H6 in tumor cell lines has been shown to reduce migratory ability, proliferation and invasiveness, suggesting it to be important for malignancy (160). With regards to future immunotherapies, promising preliminary results have been observed with CARs and BiTEs targeting B7H6 (164-166). One should, however, show caution, as B7H6 can be upregulated on proinflammatory monocytes in sepsis patients, as well as upon microbial stimuli (152). The potential adverse effects during anti-B7H6 therapy in a cancer patient, should the patient simultaneously acquire an infection, could be devastating as B7H6+ innate cells would also become targets.

It is surprising that so many tumors express high surface levels of B7H6, as it makes them susceptible targets for NK cells. Even though shedding has been observed in several tumors and tumor cell lines, this is not a universal mechanism. Why, then, is B7H6 so widely expressed in malignant tissues and tumor cell lines? The carcinoma cell lines we have investigated originated from tumors that have successfully escaped

**Figure 6.** The Ig domain of NKp30 contains a highly conserved hydrophobic area rich in glycine residues. A. Amino acid sequence alignment of a section of the NKp30 Ig domain (membrane proximal part) from the indicated mammalian species. Grey area marks the hydrophobic region. Dashes represent identical amino acids compared to human. B. Ribbon (left) and wire (right) structure prediction of the NKp30 Ig domain. Pink marks the highly conserved hydrophobic, glycine-rich area.

### B7H6 as a tumor ligand

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immune surveillance, in spite of their expression of B7H6. As mentioned previously, we did not observe any shedding from B7H6+ cell lines, suggesting that they survive in vivo with high levels of B7H6. Homodimerization of B7-1 but not B7-2 has been shown to be present both in soluble form as well as on the cell surface, and is thought to be the reason why the former binds with higher avidity to its receptors (237-239). As with dimerization on the CD28 receptor side discussed earlier, the thought of heterodimerization of B7 ligands is intriguing. Could B7H6 engage in cis with other molecules, either of the B7 family or others, on the tumor cell to achieve different binding properties such as the ability to engage an inhibitory receptor? In Paper II we investigated the expression of several B7 family members on cancer cell lines, but none of the tumor cells expressed ligands for inhibitory CD28 proteins. On the contrary, although not statistically significant, we did observe a tendency towards co-expression of ICOS-L and B7H6, both ligands for activating receptors.

Another feature of B7H6 not greatly studied is the properties of its cytoplasmic tail. The intracellular domain of human B7H6 is very long compared to the intermediate and short tails observed in cattle and rat, and there is little sequence similarity between the three species. Several putative signaling motifs have been identified in human B7H6, including an ITIM-like motif (SAYTPL), as well as putative Src-homology 2- and 3-binding domains (16). Although functional roles for these motifs have not been confirmed, a hypothesis could be that these motifs mediate signaling that is beneficial to the tumor cell.

**Experimental animal models for NKp30**

Rodents, for many reasons, are preferred species for experimental cancer and immunology research. Laboratory mice (Mus musculus) lack both B7H6 and NKp30 and Mus caroli express NKp30 but still lack a functional B7H6 gene. Our finding that B7H6 is a functional gene in the rat, and encodes a functional ligand for rat NKp30, puts the rat in position as the preferable experimental model to study both NKp30 and B7H6 biology. Looking at the NK receptor field as a whole, rodents are not optimal model systems. In rat and mouse, MHC class I recognition is carried out by the Ly49 receptor family, and only one (rat) or two (mouse) KIR loci of uncertain functional significance are present (240, 241). In the human, Ly49 only exists as a pseudogene (242) whereas the KIR family is expanded. Furthermore, although mouse CD94/NKG2A binds the HLA-E orthologue Qa-1, the signaling mechanisms of rodent CD94/NKG2 are differently organized than in other mammals (73, 243). Other differences include the KLRE/I receptor family, not present in the human (244, 245) and the expanded NKR-P1 and Clr families in rodents (246, 247). Furthermore NKp44 found in human but not in rodents (89).

Despite these differences, the rat should be a good model to study the B7H6-NKp30 interaction. An Ncr3−/− rat would enable investigation of the in vivo role of NKp30 in
experimental models for cancer, infection and autoimmunity. Targeting of $B7h6$ could help determine its role in cancer immunosurveillance as well as in general tumor development, tumor cell migration and invasiveness in an \textit{in vivo} setting. The HCMV virus has been reported to express proteins that downregulate surface expression $B7H6$ by unknown mechanisms (153, 154). Assuming a similar function is encoded in the rat CMV genome, the functional importance of such a mechanism for the outcome of CMV infection could be investigated.

One disadvantage of the rat is its lack of NKp30-S splicing signals. In confirmation of this, we have not observed a shorter splice version in rat RT-PCR experiments of several cell lines, primary cells and tissues. Secondly, semi-quantitative RT-PCR analysis of a panel of tissues from the PVG and DA rat suggested that $B7H6$ is widely expressed in non-malignant tissues, including spleen, muscle, liver and heart. However, in the absence of monoclonal antibodies for rat $B7H6$, we do not know whether this is translated and present at the protein level. We are therefore in the process of making mAbs against rat $B7H6$. 
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