Proteolytic shedding of NKG2D ligands from tumour cells

Proteolytische Freisetzung von NKG2D Liganden durch Tumorzellen

DISSERTATION

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Preface

All four chapters in the “results and discussion” section of this thesis have been published before or have been submitted for publication. At the beginning of each chapter, it is indicated which experiments were done by the author of this thesis and who else contributed to the presented work.

1 Introduction

1.1 The immune system

The immune system is a complex network of specialised cells and organs. The main function of the immune system is to protect the organism from invading pathogens but also from altered cells such as tumour cells. In most cases our immune system is able to control infections. This control is based on the ability of the immune system to distinguish between self, altered-self and foreign. Non-self substances invading the body can trigger the immune system. These substances which can be, for example parts of foreign cells, bacteria or viruses are called antigens (antibody generating). These antigens contain epitopes recognised by the immune system. Antigenic peptides are presented to the immune system by a specific set of self markers, the major histocompatibility complex (MHC) molecules which are expressed by nearly all normal body cells. The phenomenon that the immune system does not react against "self" structures is called immunological tolerance. Defects in this tolerance result in autoimmunity. The immune system is also able to recognise alterations on the surface of malignant "self" cells. This phenomenon is called tumour immunosurveillance.

The immune system is composed of two major parts, the adaptive and the innate immune system. The innate immune system is our first line of defence against pathogens and infectious agents. The elements of the innate immune system include anatomical barriers, soluble molecules and cellular components. Cellular components of the innate immune system are granulocytes, macrophages, monocytes, mast cells and natural killer (NK) cells. These cells are able to eliminate invading pathogens without prior sensitization or activation. Characteristics of the innate immunity are that the defence mechanisms are for the most part constitutively present and ready to be mobilised upon infection. In addition, it is not antigen specific, but recognises a variety of organisms due to certain surface molecules. Recent studies have shown that NK cells participate also in cancer immunosurveillance. In the following chapter NK cell biology is described in detail. The adaptive immune system is the second line of defence. In contrast to the innate immune system, it needs prior sensitization to develop an antigen-specific immune response. In addition, it has a memory and can protect more efficiently

against re-exposure to the same pathogen. The two parts of the immune system have distinct features, but they also influence each other.

1.1.1 The history of NK cells

In 1975, NK cells were first identified in mice as a distinct subpopulation of lymphocytes responsible for the "unspecific" and MHC-unrestricted cytolytic background activity. At this time they were described to be able to kill tumour cells without prior sensitization [**1-4**].

In 1985/6, Kärre, Ljunggren and colleagues found that NK cells selectively reject syngeneic MHC class I-deficient tumours efficiently in contrast to the MHC class I positive variant [**5,6**]. This MHC class I-dependent recognition was described as "missing self" hypothesis [**7**] which means that healthy autologous cells with an adequate self MHC class I expression are protected from NK cytotoxicity due to their MHC class I expression. In contrast, when cells lose the MHC class I expression, e. g. after viral infection or malignant transformation [**8,9**], NK cells do not receive any inhibitory signal. These MHC class I-deficient cells are then attacked by NK cells. These findings postulated the existence of MHC class I-specific inhibitory receptors on NK cells which subsequently were described in the early 1990s. But the "missing self" hypothesis failed to explain, why non-malignant cells with low or no MHC class I expression (such as human erythrocytes expressing no MHC class I molecules or resting cells with low expression of MHC class I molecules) are spared by NK cytotoxicity and why tumour cells were killed which express sufficient amounts of MHC class I molecules.

In the late 1990s, after the molecular characterisation of several activating NK receptors, the "induced self" recognition model was introduced [**10-12**]. This model expanded the "missing self" model in the way that NK cells are not only triggered as a consequence of loss of inhibitory signals, but that they also require activating signals. It explains that the induced expression of activating ligands in transformed or stressed cells such as tumour cells or virus-infected cells is necessary to activate NK cells.

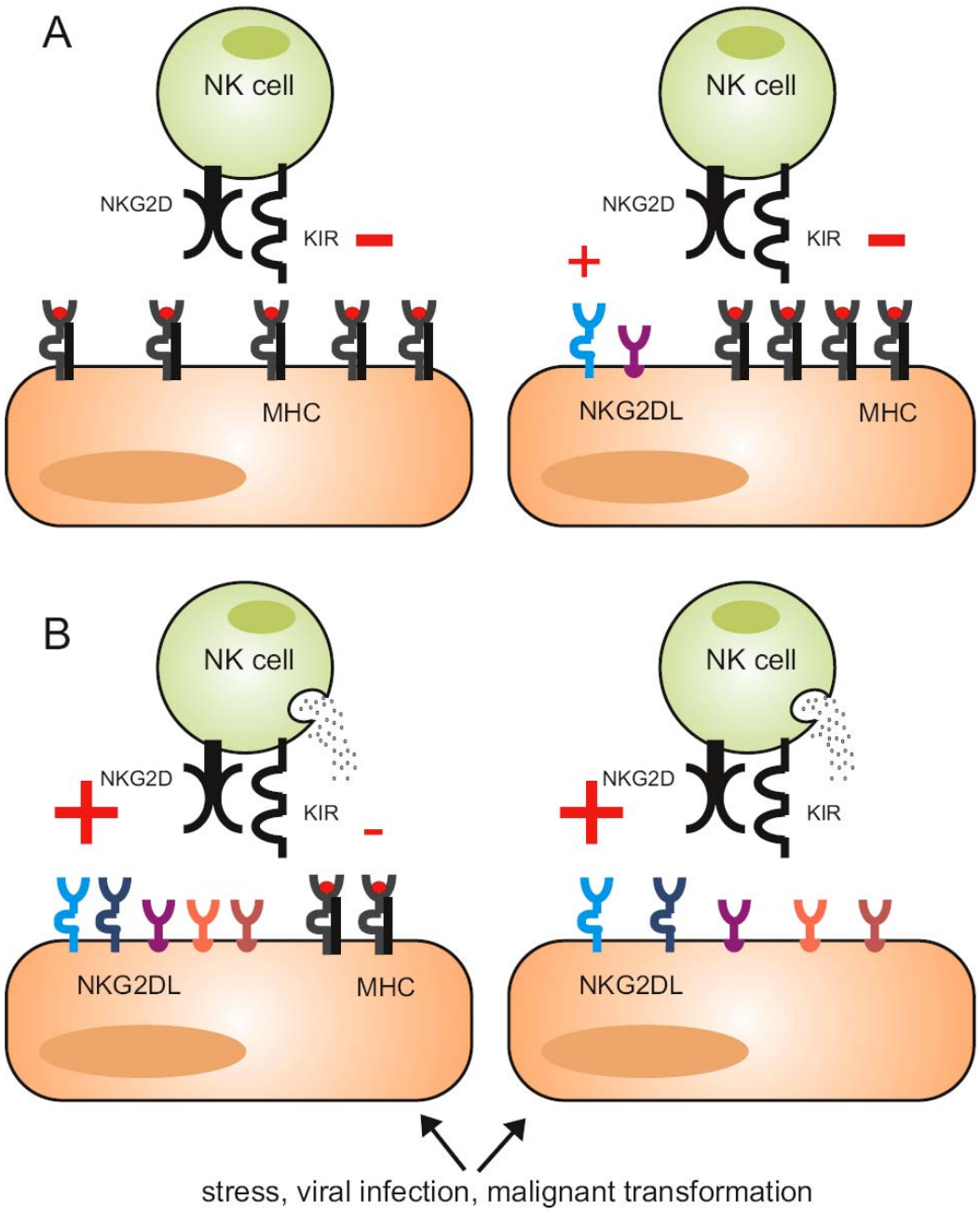

Figure 1.1. NK cell inhibition and activation. (A) A normal cell with adequate MHC class I expression and no or only low expression levels of NKG2D ligands leads to the inhibition of NK cells. This mechanism protects healthy cells from lysis by NK cells. **(B)** Upon viral infection or malignant transformation, cells may down-regulate MHC class I molecules on the cell surface and the expression of NKG2D ligands is induced. Subsequently, NK cells get strong activating signals together with low or no inhibitory signals leading to activation. After activation, NK cells release lytic granules containing perforin and different granzymes which lyse the target cell. KIR, killer immunoglobulin-like receptor; MHC, major histocompatibility complex; NK, natural killer; NKG2DL, NKG2D ligand.

Altogether, the reactivity of NK cells is determined by a balance between activating and inhibitory signals [**13**]. Normal cells express MHC class I molecules and no or only few

activating NK cell ligands resulting in an inhibition of NK cells, whereas altered or stressed cells activate NK cells in the absence of MHC class I molecules and the induced expression of activating ligands. These mechanisms are illustrated in **Figure 1.1**.

1.1.2 NK cell characteristics

In humans, NK cells are defined as $CD3^{-}CD56^{+}$ lymphocytes and they represent about 15% of all peripheral blood lymphocytes. They can be divided into two major subpopulations, $CD56^{dim}$ and $CD56^{bright}$ cells. The $CD56^{dim}$ population predominates in the blood (about 95% of NK cells) and at sites of inflammation. In addition, this population is characterised by a high cytotoxic activity and expression of killer cell immunoglobulin (Ig)-like receptors (KIR) and Fc gamma receptor III (CD16). The $CD56^{bright}$ cells are mainly cytokine producers with low cytotoxicity and no or low CD16 expression and predominate in lymph nodes (about 75% of NK cells) [**14-16**]. They are considered to represent a precursor stage of terminally differentiated $CD56^{dim}$ NK cells. CD56 is not expressed in mice. But similar to human NK cells murine NK cells can be classified according to the expression level of the tumour necrosis factor receptor superfamily (TNFRSF) member CD27. $CD27^{high}$ NK cells are cytokine producers and predominate in lymph nodes [**17,18**] but they also have a high cytolytic potential. A new suggestion is to define human and mouse NK cells by their expression of the activating NK receptor NKp46 which is almost exclusively expressed by NK cells [**19**].

The main effector mechanism of NK cells is, as their name implicates, cell-mediated cytotoxicity. Upon activation NK cells release their cytotoxic granules that contain perforin and different granzymes resulting in a perforation of the target cell and subsequent apoptotic death induced by the granzymes [**20,21**]. But there are also some members of the TNFRSF involved in the killing of sensitive targets such as tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL) and TNF receptor (TNFR)-1 [**20,21**]. The cytokines interleukin (IL)-2, IL-12, IL-15 and interferon (IFN)-α/β produced by other lymphocytes increase the activation of NK cells and augment their cytolytic activity against tumour cells [**22**]. After activation, NK cells also produce certain cytokines and chemokines like IFN-γ, granulocyte macrophage

colony-stimulating factor (GM-CSF), TNF, monocyte chemotactic protein (MIP)-1α and RANTES (regulated upon activation, normal T cell expressed and secreted) [**23-25**]. Information from *in vivo* studies indicates that IFN-γ derived from NK cells is of major importance for shaping an immune response [**24**].

1.1.3 NK cell receptors

In contrast to T and B lymphocytes, NK cells do not recognise foreign antigens, but an altered cell surface of "self" cells. NK cells do not have the possibility of genetic recombination to generate antigen-specific receptors, but they have a large repertoire of germ-line encoded inhibitory and activating receptors. These receptors mainly belong either to the immunoglobulin superfamily (IgSF) or to the C-type lectin superfamily (CLSF). Many Ig-like NK receptors like the killer-cell Ig-like receptors (KIRs), the Ig-like transcripts (ILTs), or the natural cytotoxicity receptor (NCR) NKp46 are encoded in the leukocyte receptor complex (LRC), whereas the C-type lectin-like receptors (CTLRs) are encoded in the natural killer gene complex (NKC) [**26,27**].

1.1.3.1 Inhibitory receptors

The major inhibitory NK receptors are members of the KIR family in humans, the C-type lectin-like Ly49 receptors in mice and CD94/NKG2A (natural killer group 2, member A) lectin-like receptors expressed both in humans and mice [**28-30**]. The KIR family consists of at least 15 functional genes that are highly polymorphic and that are expressed on overlapping NK cell subsets and a subset of memory T cells [**31-34**]. They are described to recognize the classical human MHC class I molecules, human leukocyte antigen (HLA)-A, -B and -C. The number and type of KIR genes present in different individuals varies significantly resulting in a different and specific KIR expression pattern in each individual.

The mouse Ly49 receptors were the first identified MHC class I inhibitory receptors [**35,36**]. The Ly49 genes are polymorphic and, similar to the KIRs, expressed on overlapping NK cell subsets and memory T cells. The receptors are specific for allelic subsets of MHC class I molecules [**33,36**].

The heterodimer NKG2A/CD94 binds to the non-classical MHC molecule HLA-E in humans and to Qa-1^b in mice, and is a broad detector of MHC class I expression [**37,38**]. NKG2A/CD94 can be detected on about 50% of the NK cells and on a subset of memory $CD8^+$ T cells.

After ligand binding to inhibitory receptors the tyrosine of the immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic tail of the receptors is phosphorylated. This leads to a recruitment of Src homology 2 (SH2) domain-containing phosphatases such as SHP-1 or -2 (SH2-containing protein tyrosine phosphatases) causing an inhibition of NK cells [**29**]. Each NK cell normally expresses at least one inhibitory receptor which is randomly chosen out of the receptors encoded in the germ-line. If developing NK cells do not receive any inhibitory signal, but express MHC class I specific inhibitory receptors they become hyporesponsive [**39,40**].

1.1.3.2 Activating receptors

In humans, important activating NK receptors are the CTLR NKG2D and the NCRs NKp30, NKp44 and NKp46 [**41,42**]. The NCRs are almost exclusively expressed on NK cells. NKp46 and NKp30 are expressed on all NK cells, whereas NKp44 expression is restricted to activated NK cells [**43**]. The self ligands for the NCRs remain still unknown, but it was shown that NKp46 and NKp44 bind to the hemagglutinins of influenza virus [**44**] and other viral structures [**45**]. Blocking studies with anti-NCR antibodies revealed the important role of these receptors in the lysis of tumour cells [**46**].

NKG2D is one of the most important activating NK receptors. Its ligands are inducibly expressed after cell stress, viral infection and malignant transformation. NKG2D and NKG2D ligands (NKG2DL) will be discussed later in detail.

The Fc gamma receptor III (CD16) plays also an important role in NK cell activation because it mediates antibody-dependent cellular cytotoxicity (ADCC) [**14,47**].

There exist several other activating NK receptors, but their immunological function and signalling pathways are mostly insufficiently understood. The leukocyte adhesion molecule DNAX accessory molecule-1 (DNAM-1), also known as CD226, recognises ligands on tumour cells, the poliovirus receptor (CD155) and Nectin-2 (CD112) [**48**]. 2B4, another activating NK receptor, binds to CD48 which is up-regulated on cells

infected with Epstein-Barr virus and highly expressed by cells of the haematopoietic lineage [**49**]. The CTLR NKp80 binds to the genetically linked, myeloid specific CTLR AICL [**50**]. Other surface molecules which are implicated in NK cell activation and tumour cell lysis are NTB-A, CD18/CD11 (β_2 integrins), CD2 and the toll-like receptors (TLR) [**51-53**].

Most of the activating receptors of NK cells have no signalling motif in their cytoplasmic tail. Instead they associate with adaptor molecules via charged amino acids in their transmembrane regions. CD16 [**54**], NKp30 [**55**] and NKp46 [**56**] signal via association with CD3ζ and FcεRIγ. NKp44 [**43**] binds to DNAX activating protein of 12 kDa (DAP12) which contains an immunoreceptor tyrosine-based activation motif (ITAM) sequence. Upon activation it becomes phosphorylated and recruits zeta-chain-associated protein of 70 kDa (ZAP70) and Syk.

The KIR, Ly49 and NKG2 families, mainly consisting of inhibitory receptors, also have some activating members binding to MHC class I molecules [**57-59**]. Examples are KIR2DS and KIR3DS in humans, Ly49D and Ly49H in mice and CD94/NKG2C in humans and mice. These receptors have, in contrast to the inhibitory receptors, no ITIM motif but charged amino acids in their transmembrane region to recruit the ITAM-bearing adapter molecule DAP12 [**13,41**]. Surprisingly, these activating receptors bind to the same ligands as their inhibitory counterparts but their affinity to MHC class I molecules is lower [**13,60**]. The immunological relevance of these receptors remains to be elucidated.

1.1.4 The activating NK receptor NKG2D

NKG2D is a homodimeric C-type lectin-like activating receptor encoded in the NKC on chromosome 6 in mice and 12 in humans [**10,42**]. NKG2D is evolutionary conserved in contrast to its ligands. It is genetically linked to the NKG2 receptor family [**61**] but NKG2D has no significant similarity to other NKG2 members and should be considered as a distinct receptor due to its unique biology. An interesting feature of NKG2D is the multitude of ligands, whereas other NK receptors have only one or two binding partners. The homodimer of NKG2D binds to the α_1 and α_2 domain of its ligands as shown in **Figure 1.2** for the binding to ULBP3.

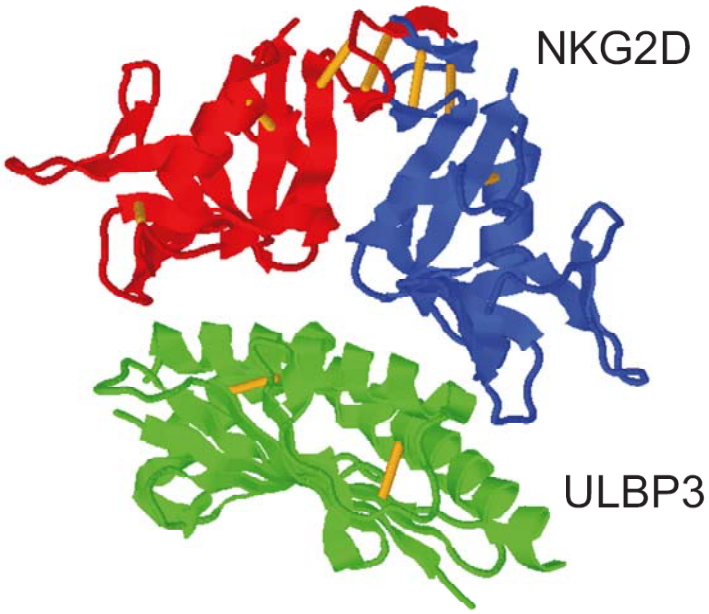

Figure 1.2. Crystal structure of NKG2D binding to its ligand ULBP3. The human NKG2D homodimer (in blue and red) binds to the α_1 and α_2 domain of the human NKG2D ligand ULBP3 (in green). The structure was created using the software proteinexplorer (http://www.proteinexplorer.org), PDB file, 1kcg [**62**]. ULBP3, UL16-binding protein 3.

NKG2D is expressed on almost all NK cells, $CD8^+$ αβ T cells and γδ T cells, but only on a few $CD4^+$ αβ T cells in humans [**10**] which can be expanded in patients with rheumatoid arthritis or tumours [**63,64**]. In addition, NKG2D is expressed on subsets of natural killer T (NKT) cells.

In mice, NKG2D expression on T cells differs from humans since only a portion of γδ T cells, activated and memory $CD8^+$ T cells express this receptor [**65,66**], whereas all mouse NK cells express NKG2D.

NKG2D expression can be modulated by different cytokines. On human NK cells it is up-regulated by IL-15 [**67,68**]. Transforming growth factor (TGF)-β [**69,70**] and IL-21 [**71**] were shown to down-modulate NKG2D expression.

At the moment, NKG2D is the best-characterised activating NK receptor. It associates via charged amino acids in the cytoplasmic tail with the adapter protein DNAX activating protein of 10 kDa (DAP10). Association with DAP10 can trigger cytotoxicity in NK cells. In mice, an alternative splice variant of NKG2D, NKG2D-S, can also associate with DAP12 [**72,73**] triggering cytotoxicity and cytokine release. DAP10 has in contrast to DAP12 no ITAM motif, but a cytoplasmic YxxM motif, which recruits phosphatidylinositol 3-kinase (PI3K) after phosphorylation at its tyrosine residue eventually resulting in the activation of NK cell cytotoxicity [**74**].

1.1.5 NKG2D ligands

NKG2D has a multitude of ligands. In humans, two families of NKG2DL exist, the MHC class I-related molecules A and B (MICA and MICB) and the UL16-binding proteins (ULBPs). MICA and MICB are non-classical MHC molecules, closely related to each other and encoded by tandem genes near the *HLA-B* locus [**10**]. The MIC proteins consist of MHC class I-like α1, α2 and α3 domains **(Figure 1.3)**, but they do not associate with β_2-microglobulin and they are TAP-independent [**75,76**].

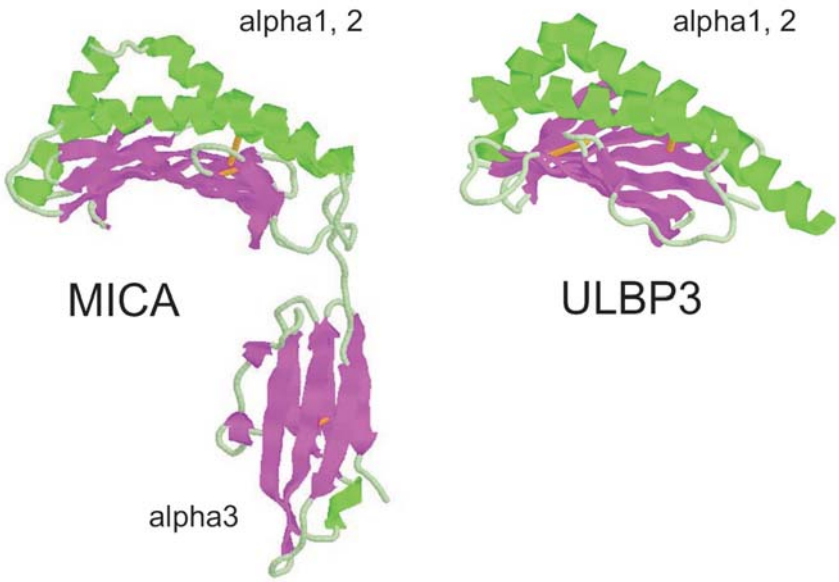

Figure 1.3. Crystal structure of NKG2DL. The structure of MICA (left) as an example for the family of the MHC class I chain related (MIC) molecules consists of an α_1, α_2 and α_3 domain which are very similar to MHC class I proteins. ULBP3 as an example for the UL16 binding proteins (ULBPs) lacks an α_3 domain, but the α_1 and α_2 domains have a comparable structure to MICA. NKG2D binds to the α_1 and α_2 platform of the NKG2D ligands. The structures were created using the software proteinexplorer (PBD file MICA, 1hyr [**76**], ULBP3, 1kcg [**62**]). MICA, MHC class I chain related protein A; ULBP3, UL16-binding protein 3.

Members of the ULBP family were identified by their binding capacity to the human Cytomegalovirus (HCMV) protein UL16 [**77**] and due to their relationship to the mouse retinoic acid early inducible (Rae)1 proteins [**78**]. Therefore, they are named retinoic acid early inducible transcript (RAET)1 proteins. The ULBP gene cluster on the long arm of chromosome 6 consists of at least six functional genes (ULBP1-4, RAET1G and RAET1L) and four pseudogenes [**78-80**], however the binding of RAET1L to NKG2D needs to be confirmed. ULBPs are also members of the MHC class I family, but they

share no sequence homology with MIC molecules. They consist of an α1α2 platform domain lacking an α3 domain **(Figure 1.3)**. Some of them are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI)-anchor (ULBP1-3 and RAET1L), whereas ULBP4 and RAET1G have a transmembrane domain and a cytoplasmic tail [**62,77,81**]. The NKG2DL are highly polymorphic, especially the MIC molecules with over 70 distinct alleles identified [**79,82**]. ULBP1, ULBP2, RAET1G and MICB bind to the HCMV glycoprotein UL16 resulting in an intracellular retention, whereas MICA, ULBP3 and ULBP4 do not bind to UL16 [**77,83-85**]. MICA surface expression, except for the allelic variant MICA*08 which lacks the cytoplasmic tail, is inhibited by the HCMV protein UL142 [**86**].

NKG2DL	synonym	plasma membrane anchoring	NKG2D binding affinity [ref.]
MICA	-	transmembrane domain	0.9 μM [**87**]
MICB	-	transmembrane domain	0.8 μM [**88**]
ULBP1	RAET1I	GPI-anchor	1.1 μM [**89**]
ULBP2	RAET1H, ALCAN	GPI-anchor	?
ULBP3	RAET1N	GPI-anchor	?
ULBP4	RAET1E, LETAL	transmembrane domain	?
RAET1G	-	transmembrane domain	?
RAET1L	-	GPI-anchor	?

Table 1.1. Human NKG2DL. All human NKG2DL are listed with their synonyms and their plasma membrane anchoring. In addition, the binding affinity to NKG2D is indicated. Table adapted from [**90**]. GPI, glycosylphosphatidylinositol; MIC(A/B), MHC class I chain-related protein A/B; NKG2DL, NKG2D ligands; RAET1, retinoic acid early inducible transcript; ULBP, UL16-binding protein.

In mice, NKG2DL are the retinoic acid early inducible 1 (Rae1) proteins [**91,92**], the distantly related minor histocompatibility antigen H60 [**91,92**] and murine UL16-binding protein-like transcript 1 (Mult1) [**93,94**]. The MIC molecules have no homologues in mice. Until now, five isoforms of the mouse GPI-anchored Rae1 proteins are known called Rae1-α, -β, -γ, -δ and -ε. All mouse NKG2DL are distantly related to MHC class I molecules but do not associate with β_2-microglobulin and are TAP independent. Structurally, mouse NKG2DL are most similar to ULBPs.

NKG2DL	plasma membrane anchoring	NKG2D binding affinity [ref.]
Rae1α	GPI-anchor	690 nM [**95**]
Rae1β	GPI-anchor	345 nM [**95**]
Rae1γ	GPI-anchor	586 nM [**95**]
Rae1δ	GPI-anchor	726 nM [**95**]
Rae1ε	GPI-anchor	?
H60	transmembrane domain	26 nM [**95**]
MULT1	transmembrane domain	6 nM [**93**]

Table 1.2. Mouse NKG2DL. All mouse NKG2DL are indicated with their plasma membrane attachment and their binding affinity to NKG2D if known. GPI, glycosylphosphatidylinositol; MULT1, murine UL16-binding protein-like transcript 1; NKG2DL, NKG2D ligands; Rae1, retinoic acid early inducible 1.

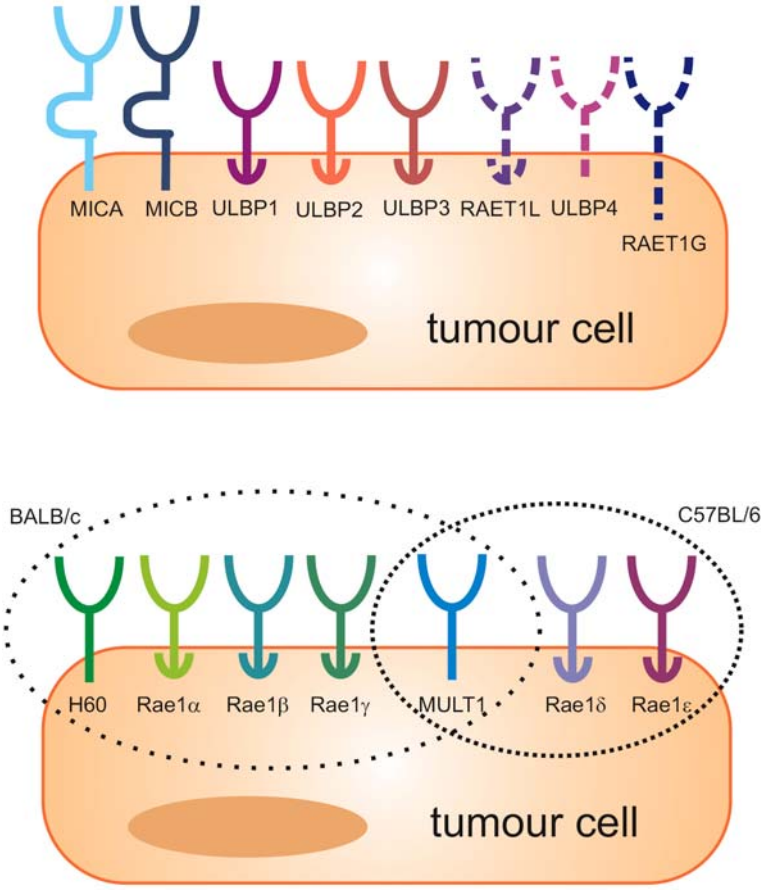

Figure 1.4. Human and mouse NKG2DL. In the upper panel the human NKG2DL are shown. The members of the MHC class I chain-related proteins MICA and MICB and the UL16-binding proteins ULBP1-4, RAET1G and RAET1L. In the lower panel, the mouse NKG2DL Rae1, H60 and MULT1 are shown. They are differently expressed by the mouse strains BALB/c and C57B1/6. MIC(A/B), MHC class I chain related protein A/B; MULT1, murine UL16 binding-like transcript 1; NKG2DL, NKG2D

ligand; Rae1, retinoic acid early inducible 1; RAET1, retinoic acid early inducible transcript 1; ULBP, UL16-binding protein.

All NKG2DL form a similar tertiary structure as evident from crystallographic analyses **(Figure 1.3)** allowing them to bind in a comparable way to the NKG2D homodimer [**62,76,96**]. However binding affinities of the different NKG2DL to NKG2D vary over a broad range [**78,95,97**].

1.1.6 NKG2D ligand expression

NKG2D ligands are normally not expressed or only at low levels in healthy tissues. But after heat shock, toll-like receptor (TLR)-signalling, viral infection, DNA damage and ultraviolet (UV) radiation they are up-regulated [**75,98,99**]. MICA and MICB expression is induced by stress stimuli and they are often expressed on the cell surface of epithelial tumours, on melanoma cells, hepatic carcinomas, and some haematopoietic malignancies, but they are normally absent from the healthy tissue [**75,100-102**]. Their normal tissue expression is restricted to the intestinal epithelia probably due to the neighbouring bacterial flora [**75**]. MIC molecule expression was also detected on LPS-stimulated macrophages, activated T cells, HCMV-infected fibroblasts, mycobacteria-infected dendritic cells and tissues exposed to autoimmune attack. This shows that MIC molecules are induced by many stimuli [**63,75,98,103-105**]. In contrast to the MIC molecules, ULBP transcripts are detectable in many tissues [**77,79**]. But their protein expression pattern has to be further addressed. They are frequently over-expressed in primary tumours, including colon, lung, stomach and breast carcinomas [**22**]. ULBP1-3 expression was also detected on B cells and, donor-dependent, on monocytes and granulocytes [**106**].

In mice, expression of Rae1 molecules was first described in brains of mouse embryos [**107**]. They may be involved in embryonic development as they are expressed only in early embryos especially in the brain, whereas in adult tissues they are mostly silent [**91,107,108**]. Murine NKG2DL are expressed on thymocytes and on Concanavalin A-activated splenocytes of BALB/c mice [**92**] and Rae1 proteins were induced on macrophages through TLR stimulation [**109**]. Rae1 and H60 proteins were shown to be absent in normal skin, but transcripts were induced by treatment with phorbol-12-myristate-13-acetate (PMA) and were present in papillomas and carcinomas of tumour-bearing mice [**110**].

Generally, NKG2DL in humans and mice are up-regulated in non-tumour cell lines by genotoxic stress and activation of the major DNA damage checkpoint involving ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) kinases [**111**]. In addition, the expression of ligands on tumour cell lines could be suppressed by down-regulation of ATM indicating that ligand over-expression in tumour cells may be due to chronic DNA damage response [**99**].

The expression pattern of NKG2DL suggests that they signal danger to the immune system. This signalling activates the immune system and should lead to protection from infections or "altered" cells developing into tumours. The question why there are so many different NKG2DL has to be addressed further. There are some possible explanations for the multitude of different ligands. One explanation is that the diversity has been driven by a competition between the immune system and pathogens. Another possibility is that cancer was responsible for the diversity. There is also experimental evidence that the binding affinities are different for NKG2D and also tissue-specific functions have to be considered [**90**].

1.1.7 Implications of NK cells for cancer immunosurveillance

In humans, most evidence for a role of NK cells in tumour surveillance is derived from correlative studies. In an 11-year follow-up study, it was found that a low NK-like cytotoxicity of peripheral blood lymphocytes correlates with an increased risk for cancer [**112**]. The general NK cell: tumour cell ratio found in tumours is too low to induce NK cell elimination of tumour cells *in vitro*, but possibly, *in vivo* fewer numbers of NK cells are sufficient especially against smaller tumours [**113**]. NK cells are not found in large numbers in advanced human neoplasms indicating that they normally do not home efficiently in malignant tissues. After activation with cytokines, NK cells are much more efficiently able to infiltrate tumours [**114**]. The infiltration of tumours with NK cells has been shown to be a positive prognosis marker in different carcinomas [**115-117**]. It is shown that myeloid leukaemia patients have a remarkable increase in survival and protection from relapse when they receive alloreactive NK cells in the course of allogeneic haematopoietic stem cell transplantation. This requires that the patient lacks HLA class I ligands for the inhibitory KIR receptors of donor NK cells [**118,119**].

In mice exist many reports indicating a role of NK cells in the immunosurveillance of tumours. Most of these studies were performed by implanting syngeneic tumour cells in mice. These mice were genetically deficient either in NK cells or NK cell functions or NK cells were depleted by antibodies [**18,22**]. It was demonstrated by different groups that NK cells play a role in the growth of transplantable tumours. Depletion of NK cells in such a model leads to more aggressive tumour growth and metastasis. Ectopic expression of the murine NKG2D ligands Rae1-β and H60 in tumour cell lines result in a potent rejection by syngenic mice [**120,121**]. NK cells and $CD8^+$ T cells are responsible for the rejection and the rejection is dependent on functional NKG2D [**121-123**]. Transgenic mice expressing the NKG2D ligand Rae1-ε either ubiquitously or in a tissue-specific way show a systemic down-regulation of NKG2D resulting in defects of the innate immune system. These mice have higher tumour susceptibility and immune suppressed NK cells. Sustained NKG2D ligand expression, as it is the case for tumours expressing these ligands, can lead to a systemic down-regulation of NKG2D resulting in immunosuppression [**122**]. Mice expressing MICA under the H2-K^b promoter also showed a down-modulation of NKG2D mainly due to cell-bound MICA and impacts tumour immunity as apparent in the failure of rejecting NKG2DL-expressing RMA cells [**123**]. Another strategy to improve NK cell function is administration of cytokines or chemokines such as IL-2, IL-12, IL-15 and IFN-α/β after tumour transplantation resulting in a better elimination of tumours [**18,22,24,124**]. After their activation, NK cells are able to produce certain cytokines and chemokines like IFN-γ, GM-CSF, TNF-α [**23**]. The release of IFN-γ leads to stimulation and maturation of DC via an IFN-γ dependent signal cascade to the IL-12 producing DC1 phenotype and finally in a strong and protective $CD8^+$ T cell response [**125,126**]. Experiments in mice lacking IFN-γ, perforin or recombination activating gene (RAG) 2 result in a higher incidence for tumours as compared to control mice. These results support the theory that NK cells and cytotoxic lymphocytes (CTLs) are important in tumour immunosurveillance [**127-133**]. The relevance of NK cells for controlling *de novo* tumourigenesis was demonstrated by treating NK cell-depleted mice with the chemical carcinogen methylchloanthrene (MCA). The depletion of NK cells resulted in a higher incidence for spontaneous tumours [**134**]. Another hint for the involvement of NK cells in immunosurveillance came from experiments showing that mice lacking perforin, IFN-γ, IFN-γR or signal transducer and activator of transcription (STAT) 1 establish more MCA-induced tumours compared to wildtype counterparts [**129,131,135**]. NK cells and γδ T cells are

also capable in rejecting spontaneously arising MHC class I-deficient B cell lymphomas via perforin-mediated cytotoxicity [**136**]. In another approach, mice were treated with MCA in combination with neutralising anti-NKG2D antibodies. Here, mice have a higher incidence for sarcoma meaning that NKG2D plays an important role in the protection from *de novo* tumourigenesis of MCA-induced sarcomas [**137**]. NKG2D is mainly responsible for the activation of perforin-mediated cytotoxicity as perforin-deficient mice do not show a detectable NKG2D phenotype, whereas IFN-γ- and TRAIL-deficient mice do. It has further been shown that NKG2D is involved in the tumour immunosurveillance of MHC class I positive tumours expressing the NKG2DL Rae1 compared to appropriate tumours without NKG2D ligands *in vitro* and *in vivo*. Under certain circumstances memory T cells are primed against the transplanted tumour, which eliminate tumour cells even when they do not express NKG2DL any longer [**120,121**]. Blocking of NKG2D enhances the growth and metastasis of tumours expressing endogenously or ectopically NKG2DL [**121,138**]. In experiments analysing the effects of cytokines to NK cell mediated tumour metastasis suppression it has been shown that IL-2 and IL-12 promote NK cell perforin-mediated activity mainly via the NKG2D pathway. In contrast, IL-18 functions via FasL in suppressing tumour metastasis, independently of NKG2D such as TRAIL-mediated apoptosis which is also NKG2D independent [**124**].

Taken together, these results suggest that NKG2D is critically involved in tumour immunosurveillance. Main features of this activating receptor are the broad expression on lymphocytes with the capacity for cell-mediated cytotoxicity and a large family of stress inducible ligands [**139**].

1.1.8 Tumour immune evasion

Different mechanisms are known how tumour cells evade the immunosurveillance by NK cells [**140**]. In some leukaemia cell lines the MHC class I molecules are up-regulated and provide therefore a stronger inhibitory signal to NK cells [**141**]. Patients with haematological malignancies sometimes have abnormal NK cell numbers, phenotypes and functions [**142,143,143**]. The question is why tumours develop when they express ligands for the NK cell activating receptor NKG2D. An explanation is that these tumours may survive because they are able to balance the activating and inhibitory signals to NK cells and other immune cells and are therefore tolerated by the immune

system [**144**]. Various leukaemias and other tumours secrete high amounts of soluble NKG2D ligands [**145,146**]. The surface density of the NKG2D ligands is reduced by the shedding and therefore the tumour cells are less immunogenic. Patients with epithelial tumours expressing MIC molecules have reduced expression levels of NKG2D on tumour-infiltrating and peripheral blood T and NK cells [**145**]. This effect is accompanied by up-regulated levels of soluble MICA (sMICA), soluble MICB (sMICB) and soluble ULBP2 (sULBP2) which are released by metalloproteases from tumour cells [**146,147**]. Soluble MICA causes the down-regulation of NKG2D which results in impaired NK and T cells [**145,148**]. The release of sMICA depends on the disulphide-isomerase Erp5 which induces a large conformational change before proteolytic cleavage [**149**]. Soluble MICB and sULBP2 which are found in patients with haematopoietic malignancies do not alter NKG2D expression levels *in vitro* but the NKG2D ligand shedding reduces surface densities and therefore the immunogenicity of the tumour cell [**147,150**]. Different tumour cells like leukaemia cells and glioblastomas release the potent immunosuppressive molecule transforming growth factor-β (TGF-β). It strongly down-regulates NKp30 and to a lesser extent NKG2D [**69**]. TGF-β has been shown to impair NK cell function via down-regulation of NKG2D in lung cancer and colorectal cancer patients whereas other receptors are not affected [**151**]. TGF-β is also involved in the immune escape of glioma cells by down-regulating NKG2D on NK and T cells. In addition, the NKG2DL MICA, ULBP2 and ULBP4 are down-modulated by TGF-β [**70,152**]. NKG2D and NKp46 are also down-regulated by L-kynurenine, a tryptophane catabolite generated by indolamine 2,3-dioxygenase (IDO) which has been found in various tumours correlating with tumour progression [**153**].

Taken together, soluble NKG2DL, IDO and TGF-β appear to represent important players in tumour-mediated immunosupression of NKG2D-mediated immunosurveillance and therefore are attractive targets for future immunotherapeutic strategies. A summary of these immune escape mechanisms is shown in **Figure 1.5**.

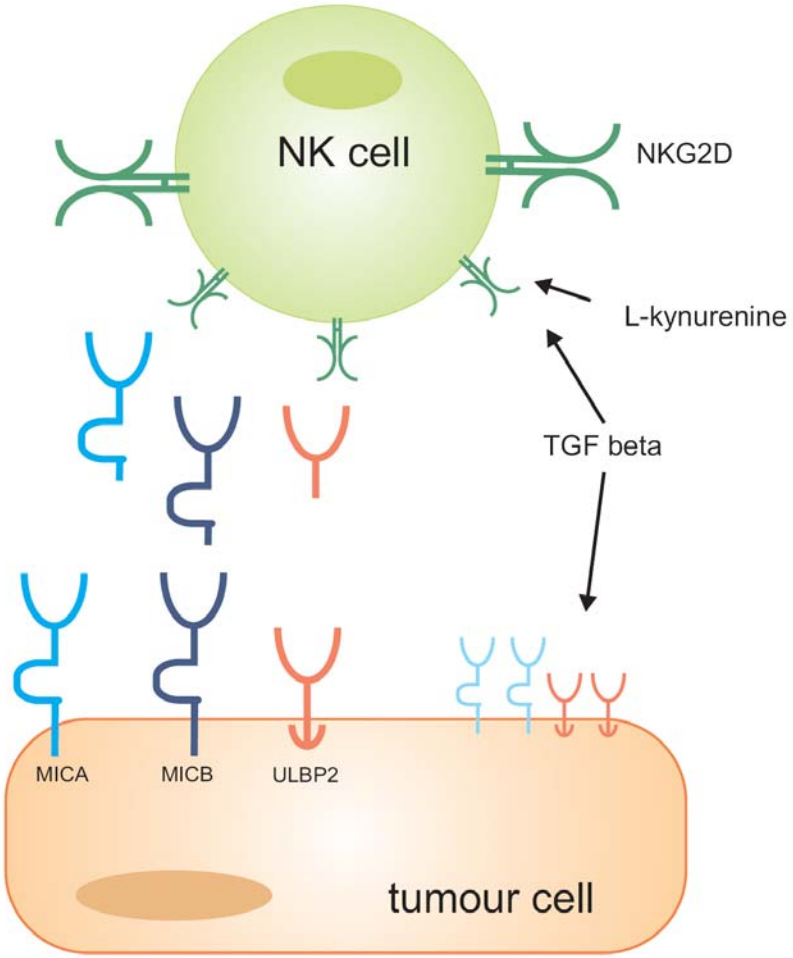

Figure 1.5. Immune evasion mechanisms of tumour cells. NK cells release soluble NKG2D ligands through proteolytic cleavage. This leads to a down-regulation of these ligands on the surface of the tumour cell. In addition, the released soluble MICA down-regulates NKG2D on the surface of NK cells. TGF-β and L-kynurenine, released by tumour cells also down-regulate NKG2D [**69,70,153**]. TGF-β can also suppress the expression of the NKG2DL MICA and ULBP2 by reducing the mRNA levels [**152**]. MICA, MHC class I chain related molecule A; NK, natural killer; ULBP, UL16-binding protein; TGF, transforming growth factor.

1.2 Metalloproteases

Secreted proteins are often derived from integral plasma membrane proteins. These proteins are post-translationally hydrolysed from the cell surface by proteases or phospholipases depending on the type of the membrane anchor of the protein. Secretion of proteins through proteolytic release is limited to type I and type II transmembrane proteins with the cleavage site generally located close to the membrane surface. The proteolytic cleavage occurs in a stalk region between the transmembrane domain and the globular extracellular domain releasing the bulk of the protein into the extracellular milieu, often in an fully functional form [**154**]. Some GPI-anchored proteins are also released by proteolysis, e. g. the folate-receptor [**155**], but more often they are released by the action of phospholipase C or D (e. g. shedding of alkaline phosphatase) [**156**]. Shedding of cell surface proteins results in a down-regulation of the protein on the cell surface and to the generation of a soluble form with identical or different properties. Receptors and receptor ligands, ectoenzymes, cell adhesion molecules and other proteins are released by the activity of different proteases from the cell surface. The majority of secretases are metalloproteases. Within this family the "a disintegrin and metalloprotease" (ADAM) proteins are the largest group [**157**]. They will be discussed in detail in the following chapter.

1.2.1 "A Disintegrin and Metalloprotease" (ADAM) proteins

1.2.1.1 Structure and properties of ADAMs

ADAM (a disintegrin and metalloprotease) proteins are multidomain proteins and belong to the superfamily of zinc proteases. This superfamily is subdivided into gluzincins, metzincins, inuzincins, carboxypeptidases, and DD-carboxypeptidases due to the primary structure of their catalytic sites [**158**]. The metzincin family consists of serralysins, astacins, matrixins, and adamalysins [**159**]. Members of the adamalysins are ADAMs, class III snake venom metalloproteases (SVMP) and ADAM-TS (ADAMs with thrombospondin motif) proteins, which can be distinguished structurally from each other **(Figure 1.6)**. Adamalysins and matrixins, including the matrix metalloproteases

(MMP), are similar in their metalloprotease domain, but the adamalysins differ by an additional integrin receptor-binding disintegrin domain **(Figure 1.6)**.

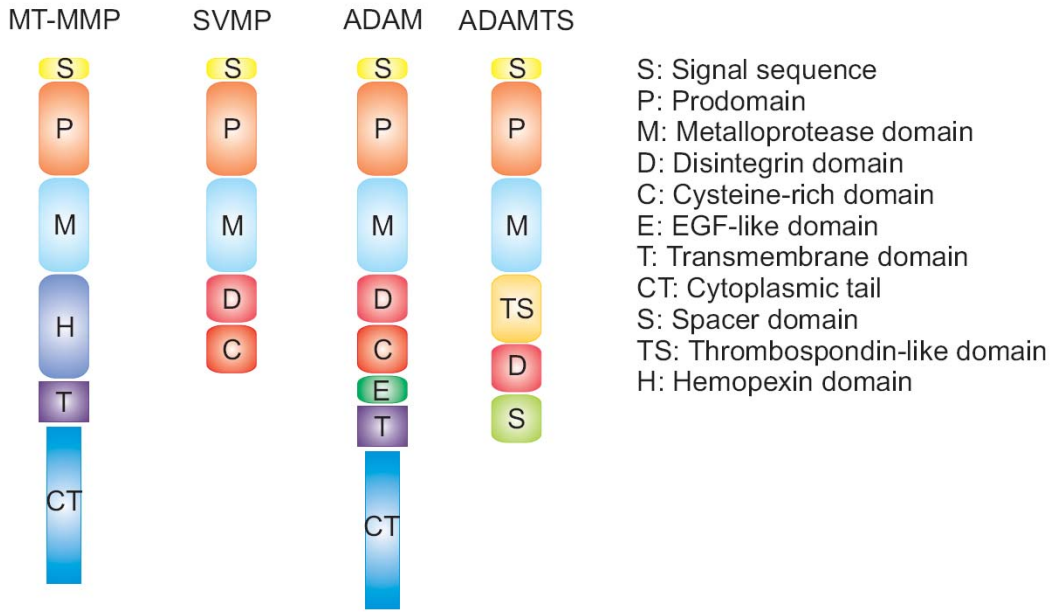

Figure 1.6. Structure of ADAMs and related proteins. The metzincin family of proteases is characterised by a metalloprotease domain with a zinc atom responsible for their proteolytical activity. The subfamily of Adamalysins consists of snake venom metalloproteases (SVMP), a disintegrin and metalloprotease (ADAM) proteins and ADAMs with thrombospondin-like domain (ADAM-TS). In contrast to the membrane-tethered matrix metalloproteases (MT-MMP) which belong to the matrixins, they contain a disintegrin domain.

The ADAMs are the largest family of membrane-tethered proteases which shed membrane proteins [**157**]. They were originally associated with sperm-egg fusion. More recently, they have been linked to other biological processes, such as cell migration and adhesion and activation of signalling pathways by shedding membrane bound cytokines and growth factors. Sometimes ADAMs are also called the MDC family, indicating the presence of a metalloprotease, a disintegrin and a cysteine-rich domain [**160**]. Up to know more than 30 members of the ADAMs are known in mammals. Some ADAMs are restricted to testis and/or associated structures (ADAM2, 7, 18, 20, 21, 29, 30 and 32) whereas other members of this family are more broadly expressed (ADAM8, 9, 10, 11, 12, 15, 17, 19, 22, 23, 28, and 33). In humans 20 *adam* genes have been identified.

ADAM	Function	Localisation	Synonyms
ADAM2	sperm-egg binding and fusion	Sperm	fertilin-β, PH-30β
ADAM7		Testis	EAP-1, GP-83
ADAM8	immune function (neutrophil infiltration, CD23 shedding)	macrophage, neutrophil	MS2, CD156
ADAM9	myogenesis, osteogenesis, cell migration, shedding of HB-EGF, TNF-p75 receptor, cleavage of APP, digestion of fibronectin and gelatin	Somatic	MDC9, MCMP, Meltrin gamma, IGF binding protein-5
ADAM10	neurogenesis, digestion of collagen IV, gelatin and myelin basic protein, cleavage of delta, APP, L1, and CD44, shedding of HB-EGF	Somatic	MADM, Kuzbanian, SUP-17
ADAM11	tumour supressor gene (?)	Brain	MDC, MDC1
ADAM12	myogenesis, osteogenesis, muscle formation, digestion of IGFBP-3 and -5, collagen IV, gelatin and fibronectin, shedding of HB-EGF	Somatic	Meltrin alpha, MCMP, MLTN, MLTNA
ADAM15	blood vessel function, expression in arteriosclerosis, digestion of collagen IV and gelatin	Somatic	Metargidin, MDC15, AD56, CRII-7
ADAM17	cleavage of pro-TNF alpha, TGF alpha, TNF-p75 receptor, ErbB4, TRANCE and HB-EGF, cleavage of APP, Notch, L-selectin and CD44	Somatic	TACE, cSVP, ADM-4
ADAM18		Testis	tMDC III(mac)
ADAM19	myogenesis, osteogenesis, formation of neuron, digestion of neuregulin	Somatic	Meltrin beta, FKSG34(gene)
ADAM20	spermatogenesis	Testis	-
ADAM21	?	Testis	-
ADAM22	?	Brain	MDC2 Alpha/Beta
ADAM23	?	brain, heart	MDC3
ADAM28	digestion of myelin basic protein and IGFBP-3, immune surveillance	testis, lung, lymphocytes, pancreas, uterus	eMDC II, MDC-Lm, MDC-Ls
ADAM29	?	Testis	svph1
ADAM30	?	Testis	svph4
ADAM32	?	Testis	-
ADAM33	mutation in bronchial asthma patients, cleavage of APP, KL-1 and insulin B chain	Somatic	-

Table 1.3. List of the human a disintegrin and metalloprotease (ADAM) family. All human ADAM family members identified until know are shown in the table. Their function, localisation and their synonyms are described. HB-EGF, heparin-binding

epidermal growth factor; TNF, tumour necrosis factor; APP, amyloid precursor protein; IGFBP, insulin-like growth factor binding protein; TGF-α, transforming growth factor-α; TRANCE, TNF-related activation induced cytokine. ADAMs with metalloprotease active site sequences are underlined. Data are from [**160,163**] and (http://www.people.virginia.edu/%7Ejw7g/Table_of_ADAMs.html).

It was shown that several ADAMs have two or more splice variants, resulting in different lengths of the cytoplasmic tail, different subcellular or tissue specific localisations or different activities [**160**]. For example, ADAM12 exists in a membrane bound form and in a shorter version without transmembrane domain secreted from the cell [**161**]. ADAM10 is also alternatively spliced and exists as a membrane bound and secreted form [**162**].

ADAMs are type I transmembrane proteins with the catalytic domain being localised on the extracellular side of the membrane. Besides that, they have a signal sequence and a prodomain at the N-terminus. The signal sequence directs the protein into the secretory pathway, whereas the prodomain inhibits the active site through a cysteine switch before activation [**164**]. This cysteine switch allows the coordination of the active site zinc atom by a conserved cysteine residue in the prodomain. The maturation of ADAMs occurs in the trans-Golgi network with the help of proprotein convertases as furin or others [**165**]. They are supposed to cleave the prodomain at a conserved Rx(R/K)R motif and after cleavage of the prodomain the zinc atom can switch the coordination to the metalloprotease domain resulting in catalytic activity [**166-168**]. But there are also some ADAM family members which may undergo autocatalytic activation; for ADAM8 and ADAM28 it was shown that an active metalloprotease domain is required for activation of the protease [**169,170**]. The prodomain has an additional function as it is required for the proper folding of the protein especially the metalloprotease domain, e.g. ADAM10 and ADAM17 proteins lacking a prodomain have no protease activity [**166,171**]. Taken together, the prodomain is important for maintaining the latency of the enzymes and it serves as an intramolecular chaperone for the active site and ensures the delivery through the secretory pathway.

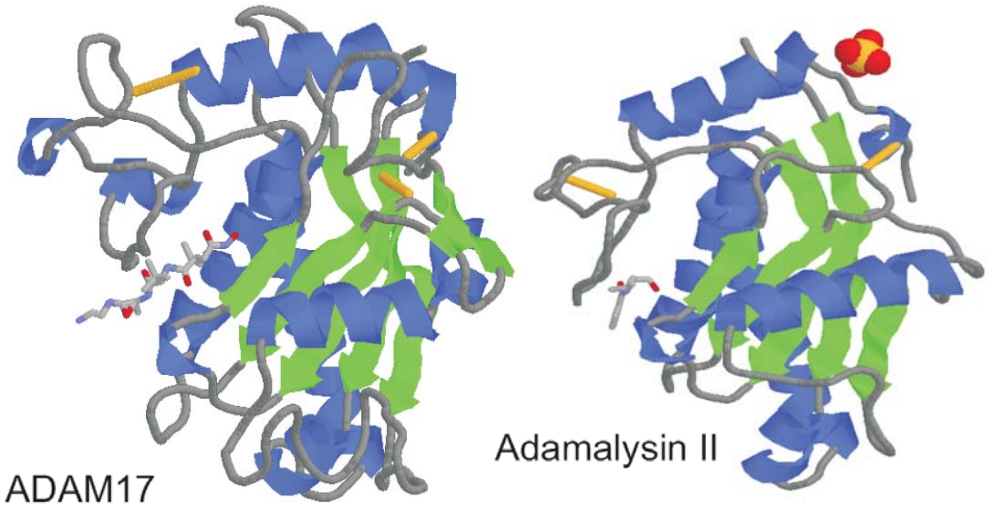

Figure 1.7. Crystal structure of the catalytic domain of ADAM17 and the snake venom metalloproteinase Adamalysin II. The crystal structure of ADAM17 (PDB file: 1bkc) is shown here with a hydroxamate-based inhibitor bound to the active site of the protein [**172**] together with the catalytic domain of the snake venom metalloproteinase Adamalysin II with a peptidomimetic inhibitor (PDB file: 2aig) [**173**]. The catalytic domains of these two proteins have very high similarities. ADAM, a disintegrin and metalloprotease. The structures were created using the software proteinexplorer (http://www.proteinexplorer.org).

All ADAMs contain a metalloprotease domain which can induce ectodomain shedding and can cleave extracellular matrix (ECM) proteins. Most ADAMs have a characteristic HExxHxxGxxH zinc-binding motif in this domain [**174**]. But about 60% of all ADAMs have mutations in this motif resulting in loss of proteolytic activity [**163,175**]. The catalytic site is highly conserved within the metzincins, but there are some differences in structure determining substrate specificity and differential sensitivity to protease inhibitors [**159**]. The structure of the catalytic domain of ADAM17 in comparison to the structure of the SVMP Adamalysin II is shown in **Figure 1.7**. In catalytically active ADAMs, three conserved histidine residues ligate the catalytic zinc atom, whereas the glutamic acid residue promotes catalysis by positioning and activating a water molecule to attack the peptide backbone of the substrate [**174**]. Twelve of the known human ADAMs (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30 and 33) contain a functional metalloprotease active site sequence indicating protease activity, although this has not been shown for ADAM20, 21, and 30 [**163**]. Surprisingly, no apparent consensus recognition sequence for cleaving membrane-anchored substrates exists. This suggests that secondary structures, the distance of the stalk region to the membrane, distal sites

and auxiliary factors contribute to cleavage-site selection of membrane-bound substrates [**157,160**].

Next to the metalloprotease domain there are three other domains: the disintegrin domain, the cysteine-rich domain and the EGF-like domain, which are suggested to promote interactions with other molecules *in vitro* and *in vivo* [**176**]. The disintegrin domain in ADAMs is about 90 amino acids long and unique among all cell surface proteins [**177**]. It is named for its presence in the SVMPs, where it is involved in binding of the platelet integrin receptor and functions as a potent inhibitor of platelet aggregation [**178**]. The disintegrin domain of the SVMPs contains a RGD consensus sequence within a 13 amino acid stretch called the disintegrin loop. ADAMs, except for ADAM15, do not have this RGD consensus sequence, but instead they have aspartic acid-containing sequences in the disintegrin loop which can bind to the α4/α9 subfamily of integrin receptors including fibronectin, vascular cell adhesion molecule (VCAM)-1, mucosal vascular addressin cell adhesion molecule (MAdCAM)-1, and tenascin-C [**160,179,180**]. All ADAMs, except for ADAM10 and ADAM17, contain this general ADAM disintegrin loop motif. Many ADAMs also have the RxxxxxxDEVF sequence in the disintegrin domain responsible for association with α9β1 integrins supporting integrin-mediated cell-adhesion [**181,182**].

The cysteine-rich and the EGF-like domain are not very well understood. Some ADAMs have sequences similar to viral fusion peptides in the cysteine-rich domain, but no experimental proof exists that they play a role in membrane fusion. It is more likely that they improve the binding capacity of the disintegrin domain and their specificity [**160**]. The cysteine-rich domain of ADAM12 has been suggested to interact with syndecans and β1 integrins [**183**], whereas the cysteine-rich domain of ADAM13 was shown to be important for specifying the protease-dependent biological response *in vivo* [**176**]. The cysteine-rich domains of ADAM10 and ADAM17 differ from those of other ADAMs [**184**]. The EGF-like domain consists of a sequence motif of about 50 amino acids with six cysteine residues and a β-sheet structure, a sequence also found in all ErbB-binding growth factors and in extracellular matrix proteins [**185**].

Most of the ADAMs have a transmembrane domain followed by a cytoplasmic tail, which differs significantly both in length and in sequence between the different ADAM family members [**160**]. The cytoplasmic tail of many ADAMs contains the PxxP motif for binding SH3 domain-containing ligands [**186-188**]. In addition, the cytoplasmic tail has phosphorylation sites for serine-threonine and/or tyrosine kinases and can be

phosphorylated under certain conditions [**160**]. This phosphotyrosines can serve as ligands for SH2 domain containing proteins [**160**]. For many ADAMs interactions with different kinases and other intercellular proteins were shown. The ability of ADAMs to release a substrate can be modulated by phosphorylation of the cytoplasmic tail or through binding of accessory proteins resulting in a different cell surface expression, an altered localisation in a specific membrane domain, or a change in the ability to cleave substrates in response to specific stimuli [**160**].

1.2.1.2 Regulation of ADAMs

ADAMs can be regulated at different stages of the protein synthesis and maturation process. A *Foxm1* knockout mouse model was shown to have a 90% reduced ADAM17 expression indicating a role of the transcription factor FoxM (Forkhead Box M) 1 in the expression of ADAM17 *in vivo* [**189**]. Other reports describe that TGF-β is involved in the expression of ADAM12 [**190**], TNF-α in the expression of ADAM8 [**191**] and intracellular reactive nitrogen species and hydrogen peroxide in the expression of ADAM9 [**192**]. The first prerequisite for ADAM activation is the cleavage of the prodomain. This occurs either in an autocatalytic manner or is accomplished by proprotein convertases as described before. The intracytoplasmic localisation of ADAMs is probably regulated through the binding of adapter proteins to the cytoplasmic tail or its phosphorylation as described above.

ADAM shedding occurs constitutively and is enhanced in response to a variety of stimuli. Protein kinase C (PKC) regulates ADAM activation. Phorbol esters such as PMA activate PKC which then activates different members of the ADAM family [**193**]; for example a direct interaction of PKCδ and ADAM9 is involved in PMA-induced heparin-binding epidermal growth factor (HB-EGF) shedding [**194**]. The association of PKCε with ADAM12 induces translocation of the protease to the cell surface [**195**]. Other studies found an involvement of the mitogen activated protein (MAP) kinase pathways in the PMA-stimulated release of HB-EGF; for example PMA stimulation of cells can lead to an increase in extracellular-signal regulated kinase (ERK) phosphorylation in combination with an increased shedding [**196**]. After PMA stimulation, the cytoplasmic tail of ADAM17 is phosphorylated via the MAP kinase

ERK which directly associates with this protease [**197**] and induces translocation of ADAM17 to the cell surface [**198**]. But it was also shown that even a mutated form of ADAM17 lacking its cytoplasmic tail is activated after PKC stimulation [**199**] indicating that there exist additional mechanisms to activate ADAM17 via PKC. Agents supporting calcium influx also induce ADAM mediated shedding. These reagents such as ionomycin lead to an increased calcium concentration in the cell and therefore to an activation of the cell. But they do not affect the PMA-induced shedding and vice versa PKC inhibitors have no effect on the shedding induced by calcium modulating agents [**200**].

Mature proteolytically active ADAMs can be regulated by the proteinacious tissue inhibitors of metalloproteases (TIMPs). In vertebrates there exist four TIMPs (TIMP-1, -2, -3, and -4) which all inhibit MMPs potently through binding to the active site in a 1:1 stoichiometric fashion [**201**]. The different TIMPs vary in their tissue expression and their ability to inhibit MMPs [**201,202**]. TIMP-1, -2 and -4 are all secreted, whereas TIMP-3 is matrix-associated [**203**]. They are endogenous regulators of MMPs but they are not entirely selective for MMPs. TIMP-3 has been shown to inhibit ADAM17 and ADAM12, whereas ADAM10 is inhibited by TIMP-1 and TIMP-3 [**204-206**]. There exist also some ADAM family members which are not sensitive to TIMPs [**207**]. It was shown that TIMP-3 inhibits tumor growth *in vivo* [**208**], but many primary tumors (gastric, pancreatic, renal, lung, breast, colon, and brain) lack detectable levels of TIMP-3 due to aberrant DNA hypermethylation correlating with disease progression [**209-211**].

1.2.1.3 ADAMs and diseases

Membrane proteins shed by members of the ADAM family are involved in the pathogenesis of diseases; examples are rheumatoid arthritis, where it was shown that TNF-α mainly released by ADAM17 is in part responsible for the inflammation [**157**], Crohn's disease and Alzheimer's disease. In cardiac hypertrophy the new ADAM inhibitor KB-R7785 blocks the shedding of HB-EGF and the transactivation of the EGF receptor during vasoactivation of cardiomyocytes has some beneficial effects when used to treat chronic cardiac hypertrophy in animal models [**212**]. ADAMs are also thought

to play an important role in development and progression of cancer because they are involved in the release of many cytokines, growth factors, their receptors and cell-adhesion molecules [**180**]. Tumour cells depend on the autocrine release of growth factors and there exist some studies describing the implication of ADAMs in the release of growth factors and in adhesion and motility of tumour cells [**160**]. In addition, they are thought to play key roles in different steps of cancer progression [**213**]. Many ADAMs are over-expressed in cancers including ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19 and ADAM28 [**163**], but for most of them the function and implication in cancer development and progression in still unknown. Up-regulated ADAM10 expression is observed for cancers of oral cavities [**214**], stomach [**215**], ovary [**216**], uterine [**216**], colon [**217**], prostate [**218,219**] and for leukaemia [**218**]. Down-regulation of ADAM10 with oligonucleotides or treatment with anti-ADAM10 antibodies reduces proliferation of carcinoma cells [**214,220**]. ADAM10 is also involved in the release of L1, a protein involved in the motility and invasion of lymphoma, lung carcinoma, and melanoma cells and which is responsible for enhanced tumour dissemination by increasing cell migration in ovarian and uterine cancer [**216,221,222**]. ADAM17 is over-expressed in cancers of the breast [**223**], ovary [**224**], kidney [**225**], colon [**226**] and prostate cancer [**227**]. Inhibition of TGF-α shedding by ADAM17 reduced the size of xenografts in nude mice [**228**] and anti-ADAM17 antibodies decreased cell proliferation in breast cancer cell lines [**223**]. In addition, it is assumed that ADAMs are involved in angiogenesis of tumours because they release membrane-tethered pro- and antiangiogenic factors [**229**].

There exist different classes of ADAM inhibitors which might be used in diseases. Synthetic small molecule inhibitors of catalysis and the proteinacious tissue inhibitors of metalloproteases (TIMPs) are more specific. Members of the small molecule inhibitors are the hydroxamate-based inhibitors that bind to the active site of ADAMs and MMPs. The crystal structure of ADAM17 in combination with a hydroxamate-based inhibitor **(see Figure 1.7)** presumably suggests that these inhibitors replace a Zn-coordinating water molecule in the active site [**172**]. Broad range metalloprotease inhibitors, such as Batimastat and Marimastat, were tested in several preclinical and clinical settings to inhibit different MMPs [**230**]. But most of them are not specific for MMPs and inhibit ADAMs to the same extent or even better. For example ADAM17 is inhibited more by Batimastat than several other MMPs [**205,231**]. The broad range metalloprotease inhibitor Batimastat (BB94) (BB94 structure is shown in **Figure 1.8**)

was used in different clinical trials. In a phase I study with patients having malignant pleural effusions, intrapleural Batimastat reduced the need for pleural aspirations, whereas in other studies intraperitoneally administered Batimastat had no positive effects [**232**]. Unfortunately, the clinical development of many broad-range metalloprotease inhibitors was not successful due to side effects, many caused by inhibition of MMPs [**233**].

ADAM	expression in cancer	function in cancer	inhibitors
ADAM8	lung, kidney, brain	migration	Batimastat
ADAM9	breast, pancreas, stomach, skin, liver, lung	cell adhesion and invasion, integrin binding	CGS 27023
ADAM10	oral cavity, stomach, ovary, uterine, colon, leukaemia, prostate	L1 shedding, cell growth and migration	GI254023X, GW280264X, INCB3619, INCB7839, XL784, TIMP-1, TIMP-3
ADAM12	brain, breast, liver, stomach, colon	HB-EGF shedding, cell growth	KB-R7785, TIMP-3
ADAM15	breast, prostate, stomach, lung	cell growth	?
ADAM17	breast, ovary, kidney, colon, prostate	TGF-α shedding, cell growth	GW280264X, INCB3619, INCB7839, TIMP-2, TIMP-3
ADAM19	brain, kidney	?	Batimastat
ADAM28	lung, breast, kidney	IGFBP-3 cleavage, cell growth	KB-R7785, TIMP-3, TIMP-4

Table 1.4. ADAM family members, their expression in human cancers and inhibitors. ADAM family members are shown which are involved in cancer development and progression. ADAMs are described with their expression and function in different cancers. Inhibitors capable of reducing ADAM activity are also shown. HB-EGF, heparin-binding epidermal growth factor; IGFBP-3, insulin-like growth factor binding protein-3; TGF-α, transforming growth factor-α; TIMP, tissue inhibitor of metalloproteases. The table was adapted from [**163**].

But there exist also inhibitors which discriminate better between ADAM family members and MMPs. ADAM9 is efficiently inhibited by CGS27023 [**234**]. GW280264X reportedly blocks proteolytic activity of both ADAM10 and ADAM17

with comparable efficiency, whereas GI254023X is about 100-fold more effective in blocking ADAM10 than ADAM17 (structures are shown in **Figure 1.8**) [**235**].

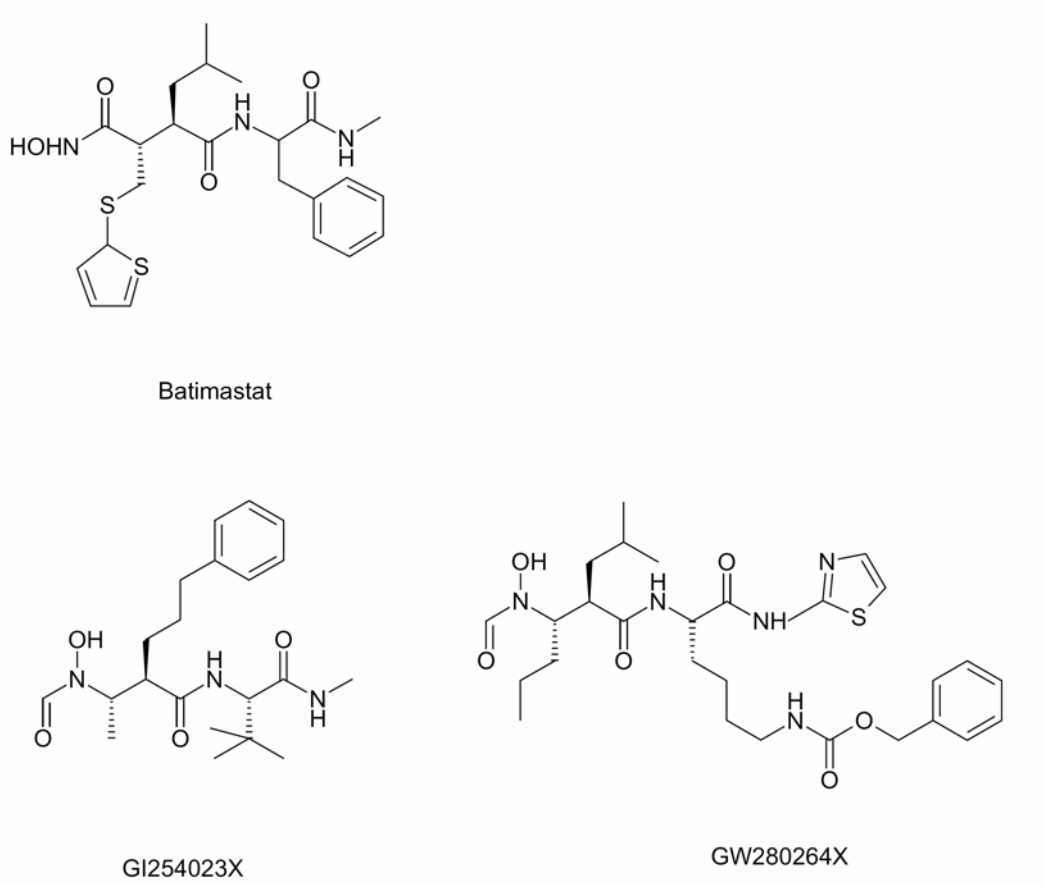

Figure 1.8. Structure of the hydroxamate-based metalloprotease inhibitors. The broad-range metalloprotease inhibitor Batimastat and the two ADAM-specific inhibitors GW280264X and GI254023X are based on the hydroxamate structure. GI254023X preferentially blocks ADAM10, whereas GW280264X has a comparable affinity to ADAM10 and ADAM17. The structure of Batimastat is adapted from [**236**] and the structures of GW280264X and GI2540023X from [**235**]. ADAM, a disintegrin and metalloprotease protein.

Some of these inhibitors are already in preclinical testing [**237,238**]. Two of them were developed to target the ErbB family, important regulators of cell proliferation and survival, via inhibition of ADAM10 and ADAM17, the main sheddases involved in the release of the ErbB ligands including EGF, TGF-α, and HB-EGF and of the respective receptors resulting in truncated constitutively active receptors [**185**]. INCB7839, reduces the release of Her-2 *in vitro* and *in vivo* and and was shown to decrease the amount of circulating Her-2 ectodomain in healthy volunteers in a phase I clinical trial [**239**]. INCB3619 is capable of preventing the functional activation of ErbB ligands through inhibition of ADAM10 and ADAM17 in a mouse model and is currently being evaluated in a clinical trial [**238**]. Another small molecule inhibitor targeting ADAMs is

XL784. It inhibits ADAM10 but also MMP2 and was originally developed as an anticancer compound. Now it is tested for administration in the pathogenesis of diabetic nephropathy due to its efficacy in preclinical models of renal failure [**240**]. These studies demonstrate that there exist some hopeful efforts to use selective ADAM inhibitors for treating different cancers and other diseases where ADAMs are involved. Apparently they only have minor side effects in contrast to their broad-range progenitors [**241**].

1.2.1.4 TACE

ADAM17 is also called TNF-α converting enzyme (TACE) which is one of the most well known members of the ADAM family. In addition, it was the first ADAM with a known substrate. ADAM17 is essential for the proteolytic release or activation of growth factors and cytokines, including epithelial growth factor ligands and TNF-α [**213**]. It releases, as the name indicates, soluble TNF-α from cells by cleavage within the extracellular domain of the membrane bound proform [**242,243**]. TACE is also involved in the cleavage of many other transmembrane proteins as TGF-α, L-selectin, the TNF receptors I and II, interleukin-6 receptor and APP which was shown in cells or mice with a deletion in the zinc-binding metalloprotease domain resulting in an inactive form of the protease [**157**]. Mice with the germ-line mutation in the metalloprotease domain of the *adam17* gene exhibit perinatal lethality or die within 2 to 3 weeks after birth [**242,244**]. Their phenotype is described with open eyelids, stunted vibrissae, and wavy hair. Histological studies revealed also defects in epithelial maturation and organization [**244**]. The structure of TACE was analysed by X-ray crystallography [**172**]. Comparison to structures of several closely-related SVMPs reveals that the catalytic domain consists of a highly conserved fold, with α-helices packed above and below a central β-sheet as shown in **Figure 1.7** [**172**]. In addition, the core structure is also very similar to that of MMP family members even if some loops have different conformations. After binding of the substrate peptide chain in the active site cleft of ADAM17, the substrate forms hydrogen bonds with the “north” site of the cleft. The catalytic zinc atom is located near the centre of the active site cleft and catalyses the cleavage of the peptide bond [**172**]. ADAM17 has a ubiquitous expression and is up-regulated in inflammation implicating that it plays a major role in cytokine biology

[**245,246**]. As mentioned before, it is up-regulated in a broad range of tumour cells. ERK, a MAP kinase, can phosphorylate the cytoplasmic domain of ADAM17 *in vitro* at threonine 735. Stimulation with PMA also leads to an increase in ADAM17/ERK association and ERK-dependent phosphorylation [**198**].

1.2.1.5 ADAM10

ADAM10 exhibits about 35% sequence identity to ADAM17 in the catalytic domain suggesting that both proteins have similar structures. It was the first ADAM family member shown to have a proteolytic activity and was originally purified from bovine brain based on its ability to cleave myelin basic protein [**247**]. The *drosophila melanogaster* homologue of ADAM10, Kuzbanian (kuz), is involved in the development of the nervous system, muscle and haematopoietic cells due to its ability to cleave Notch, a receptor that controls cell fate determination in a broad range of tissues [**248-250**]. In addition, ADAM10 seems to be involved in the cleavage of the Notch ligand Delta. ADAM10 was also shown to be responsible for the cleavage of APP as α-secretase, ephrin-A2, and HB-EGF [**160**]. ADAM10 has a broad somatic expression and is alternatively spliced resulting in a secreted and a membrane-bound form [**162**]. ADAM10 deficient mice die by day 9.5 of embryogenesis with pronounced defects in the neural and cardiovascular systems [**251**]. As mentioned before, ADAM10 is highly expressed in some types of tumours [**218**].

1.3 Aim of the thesis

The activating natural killer (NK) cell receptor NKG2D is expressed on NK cells and CD8 T cells and mediates immunosurveillance of stressed, infected and malignant cells. NKG2D ligands (NKG2DL) are expressed on malignant cells due to genotoxic stress allowing recognition and elimination of tumours by NK cells via NKG2D-mediated activation. However, there are many human tumours expressing considerable amounts of NKG2DL. This rises the question what mechanisms are employed by these tumours to prevent elimination by the NKG2D-mediated activation of NK cells and CD8 T cells. One such mechanism is thought to be the proteolytic shedding of NKG2DL by tumour cells. It was already shown that the NKG2DL MICA and MICB, type I transmembrane proteins, are released by metalloproteases from the cell surface of tumour cells. MICA shedding leads both to a reduced cell surface density of MICA on the tumour cell and to a systemic down-regulation of NKG2D on NK cells and CD8 T cells [**145-147**]. The aim of this thesis was to characterise molecular mechanisms of tumour-associated NKG2DL shedding and to identify the proteases involved in this process.

Firstly, the release of the NKG2D ligand ULBP2 should be analysed. ULBP2 is a representative of the family of GPI-anchored UL16-binding proteins. Here it should be investigated, whether ULBP2 is also shed from the cell surface of tumour cells and which mechanisms are accountable for the ULBP2 release. Further, presence of soluble ULBP2 should be analysed in sera of cancer patients.

Secondly, the yet unknown protease(s) responsible for MICA shedding should be characterised. To this aim, the MICA cleavage site and the characteristics of the MICA sheddase(s) should be analysed. The identification of the proteases responsible for MICA shedding would open the possibility to target them therapeutically by inhibitors or siRNA. This should enhance the immunogenicity of the tumour due to an increased expression of NKG2D ligands and a recovery of NKG2D-mediated reactivities of NK cells and CD8 T cells.

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2 Results and discussion

2.1 Proteolytic Release of Soluble UL16-Binding Protein 2 from Tumour Cells

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The author of this thesis performed all experiments.

2.1.1 Abstract

The MHC-class I-related ligands of the immunoreceptor NKG2D are frequently expressed by tumour cells and stimulate tumour immunity mediated by CD8 T cells and NK cells. In humans, NKG2D ligands (NKG2DL) are encoded by the MHC-encoded MIC and non-MHC-encoded ULBP families of proteins. Recently, we and others demonstrated that tumour cells release soluble MICA thereby counteracting NKG2D-mediated tumour immunosurveillance. Here, we now report that UL16-binding protein 2 (ULBP2) molecules are likewise released from tumour cells in a processed soluble form and that soluble ULBP2 (sULBP2) can be detected in sera of some patients with haematopoietic malignancies. Tumour cell-derived sULBP2 as opposed to cell-bound ULBP2 does not down-regulate NKG2D on NK cells. Unexpectedly, the glycosylphosphatidylinositol-anchored ULBP2 molecules are not released by phospholipases, but by the action of metalloproteases. Proteolytic shedding of both NKG2D ligands MICA and ULBP2 by tumour cells was strongly enhanced after PMA-treatment and paralleled by a markedly reduced susceptibility to NKG2D-mediated cytotoxicity. Shedding of MICA and ULBP2 can be blocked by the same inhibitors suggesting the involvement of related metalloproteases. Thus our data suggest that reducing NKG2DL surface densities is due to a common cleavage process executed by

metalloproteases that promotes escape of tumours from NKG2D-mediated immunosurveillance.

2.1.2 Introduction

The C-type lectin-like NKG2D receptor is expressed by most NK cells, CD8 αβ T cells and γδ T cells in humans [**1**]. In association with the adaptor protein DAP10 NKG2D transduces signals that activate or costimulate effector functions of these cytotoxic lymphocytes [**1-3**]. A peculiarity of NKG2D consists in the multitude of ligands that are not constitutively expressed but rather are induced subsequently to harmful events like genotoxic stress or infection [**3-6**]. Of note, NKG2D ligands (NKG2DL) are also frequently expressed on malignant cells, but absent from the respective benign tissues rendering the NKG2D/NKG2L-system an interesting target for tumour immunotherapy [**7, 8**]. In fact, recent studies in mice strongly support a stimulatory role of NKG2D for tumour immunity. NKG2DL expression is induced by carcinogens and genotoxic stress, and tumour cell lines transduced with mouse NKG2DL were readily eliminated in vivo due to NK and CD8 T cell activity and induced tumour immunity against the parental cell line [**6,9,10**]. Human NKG2DL belong to the two families of MHC class I-related MIC and ULBP molecules, respectively [**5**]. The MHC-encoded MICA and MICB molecules exhibit a highly restricted expression on healthy tissue in vivo, but are broadly expressed on epithelial tumours and haematopoietic malignancies [**7,8,11**]. Targeting cytotoxic lymphocytes towards MICA-expressing tumours is counteracted by proteolytic shedding of MICA molecules [**12,13**]. In addition, soluble MICA and TGFα have been reported to systemically down-regulate NKG2D expression on cytotoxic lymphocytes providing another route of escape from NKG2D-mediated [**12,14-16**]. Considerably less is known about the expression and regulation of ULBP molecules that differ from MIC molecules by the lack of an α3 domain. Like MIC molecules, ULBPs are expressed by many tumour cell lines and some haematological malignancies [**8,17**]. However, knowledge of expression of ULBP in vivo remains scarce. In contrast to MIC molecules, ULBP1-3 have been shown to be linked to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor similarly to their mouse counterparts, the RAE-1 molecules [**5,17**]. Previously, release of soluble ULBP2/ALCAN from some tumour cells in vitro has been reported [**18**], but neither the molecular mechanism of ULBP2 release nor its functional implications have been addressed. Here, we report that

ULBP2 molecules are released from tumour cells by metalloproteases and can be detected in sera of patients with haematopoietic malignancies.

2.1.3 Materials and Methods

Cells and Sera. Cell lines C1R, Jurkat, Molt4, HL60, K562 were cultured in 10% FCS-RPMI 1640, HCT116, SW756 in 10% FCS-DMEM, 293T in 10% FCS-IMDM, and C1R-transfectants in 10% FCS-RPMI 1640 with 1.8 mg G418/ml. NKL cells were cultured in 10% FCS-RPMI 1640 with 200 U/ml IL-2 (Proleukin, Chiron, CA). Human patient sera were obtained after written informed consent and with approval of the local ethics committee.

Reagents. Anti-NKG2D (clone 139), anti-NKG2D-PE (clone H106.771), anti-ULBP2 (clone 165903), polyclonal anti-ULBP2, and ULBP2-Fc were from R & D (Minneapolis, MN). Anti-mouse IgG1-PE conjugate (clone X40) and anti-CD80-FITC conjugate (clone BB1) were obtained from BD Biosciences (San Jose, CA). Soluble human PE-coupled NKG2D tetramers and anti-ULBP2 BUMO1 were produced as described elsewhere [**4**]. Rabbit anti-goat HRP conjugate was from Jackson ImmunoResearch Laboratories (West Grove, PA), anti-mouse IgG2a-HRP from Southern Biotechnology Associates (Birmingham, AL), and goat anti-mouse IgG-coated microspheres from Bangs Laboratories (Fishers, IN). Hydroxamate-based broad metalloprotease inhibitors MMP Inhibitor III (MMPI III) (Merck, Darmstadt, Germany) and Batimastat (BB94) (kind gift of Klaus Maskos, Max-Planck-Institute for Biochemistry, Martinsried, Germany) were used. BB94 was dissolved in dimethylformamide (DMF) and added as 1/200 volume to cultures. Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus cereus, Brefeldin A and PMA (Phorbol 12-myristate 13-acetate) were obtained from Sigma (St. Louis, MO).

Flow cytometry. Cells were incubated with the anti-ULBP2 BUMO1 or mouse IgG1 at 10 µg/ml and then, after washing, with goat anti-mouse-PE conjugate (1:200) as secondary reagent, or, alternatively, with PE-labelled soluble human NKG2D tetramers at 10 µg/ml. Samples were analyzed on a FACScan (BD Bioscience, San Jose, CA). Specific fluorescence intensities (SFI) were calculated by subtracting the Mean

fluorescence intensity (MFI) of the isotype control from the MFI of the specific antibody.

NKG2D down-regulation assay. NKL cells were co-cultured for 24 h with irradiated C1R transfectants at 1:1 ratio or with concentrated supernatants of C1R transfectants. Afterwards, cells were stained for NKG2D expression with NKG2D-PE or the respective isotype control. Co-cultured C1R transfectants were excluded from histogram analysis by staining with FITC-conjugated anti-CD80. After washing samples were analyzed on a FACScan.

NKG2D binding assay. Goat anti-mouse IgG-coated microspheres were incubated with 50 µg/ml anti-ULBP2 mAb BUMO1 or anti-MHC class I W6/32, respectively [**8**]. After washing, microspheres were resuspended with concentrated supernatants of C1R-ULBP2 transfectants. Then, washed microspheres were stained with PE-conjugated NKG2D or H2-Kd tetramers [**4**], respectively, and fluorescence assessed by flow cytometry on a FACScan.

ELISA. For the detection of soluble ULBP2 (sULBP2), two monoclonal anti-ULBP2 mAb binding non-overlapping ULBP2 epitopes were implemented. Plates were coated with the anti-ULBP2 mAb BUMO1 at 1 µg/ml in PBS, then blocked by addition of 50 µl of 2% BSA for 1 h at 37°C and washed. Afterwards, ULBP2-Fc (R & D) and the samples were added and the plates were incubated for 2 h at 37 °C. For analysis of patient samples, sera were diluted 1:3 in PBS prior to addition to the plates. After incubation, plates were washed and the detection mAb anti-ULBP2 (R & D) at 1 µg/ml in 1% BSA-PBS was added for 2 h at 37 °C. Plates were then washed and anti-mouse IgG2a-HRP (1:10000 in 3.25 % BSA-PBS) was added for 1 h at 37°C. Plates were then washed and developed using the Tetramethylbenzidine Peroxidase Substrate System (KPL, Gaithersburg, MD). The absorbance was measured at 450 nm. Results are shown as means with SD of triplicates. The ELISA procedure for sMICA has been previously described [**8**].

Immunoblot analysis. Samples were separated by 15% SDS-PAGE. Where indicated, samples were treated before with peptide:N-Glycanase F (PNGaseF) (New England Biolabs, Beverly, MA) for 1 h at 37 °C according to the manufacturer's instructions.

Gels were blotted to Hybond-ECL membranes (Amersham, Little Chalfont, UK), blocked with TBS containing 5% non-fat dried milk, and then analyzed with 0.1 µg/ml anti-ULBP2 serum. Binding of anti-ULBP2 was detected with a rabbit anti-goat HRP-conjugate and chemiluminescence reagent (Pierce Biotechnology, Rockford, IL).

Chromium release assay. Cytotoxicity of NKL cells against 293T cells was assessed in a 2 h 51Cr release assay. For NKG2D blockade, NKL cells were pretreated with mAb 139 and mAb139 was also added to the assay medium (10 µg/ml). Where indicated, 293T were pretreated with 10 µg Brefeldin A/ml and 100 ng PMA/ml 12 h and 11.5 h, respectively, prior to labelling with 50 µCi of 51Cr (Amersham, Freiburg, Germany) for 1 h at 37 °C, and subsequently washed three times before the assay. Calculation of % lysis: 100 x (experimental release – spontaneous release): (maximum release – spontaneous release). Data are means of triplicates.

2.1.4 Results

Tumour cell lines release soluble ULBP2

The recent description of proteolytic release of MICA from tumour cells [**12,13**] and its implications for tumour immune evasion prompted us to investigate a similar mechanism for the second family of human NKG2DL, the ULBP. We chose to investigate release of ULBP2 as a representative of the GPI-linked ULBP molecules. To this aim, we established a highly sensitive ULBP2 sandwich-ELISA detecting soluble ULBP2 (sULBP2) down to a concentration of 20 pg/ml **(Figure 2.1a)**. Using this ELISA, we analyzed culture supernatants of ULBP2-transfected C1R cells and detected high concentrations (~10 ng/ml) of sULBP2 within 16 hours of culture **(Figure 2.1b)**. Supernatants of mock-transfected C1R cells that express about 100fold less ULBP2 endogenously [**8**] as well as supernatants of ULBP1- and ULBP3-transfected gave rise to only weak signals underlining the specificity of the ELISA **(Figure 2.1b)**. In supernatants of several hematopoietic and non-hematopoietic cells lines expressing endogenous ULBP2, concentrations of sULBP2 roughly paralleled ULBP2 cell surface levels **(Figure 2.1c)**.

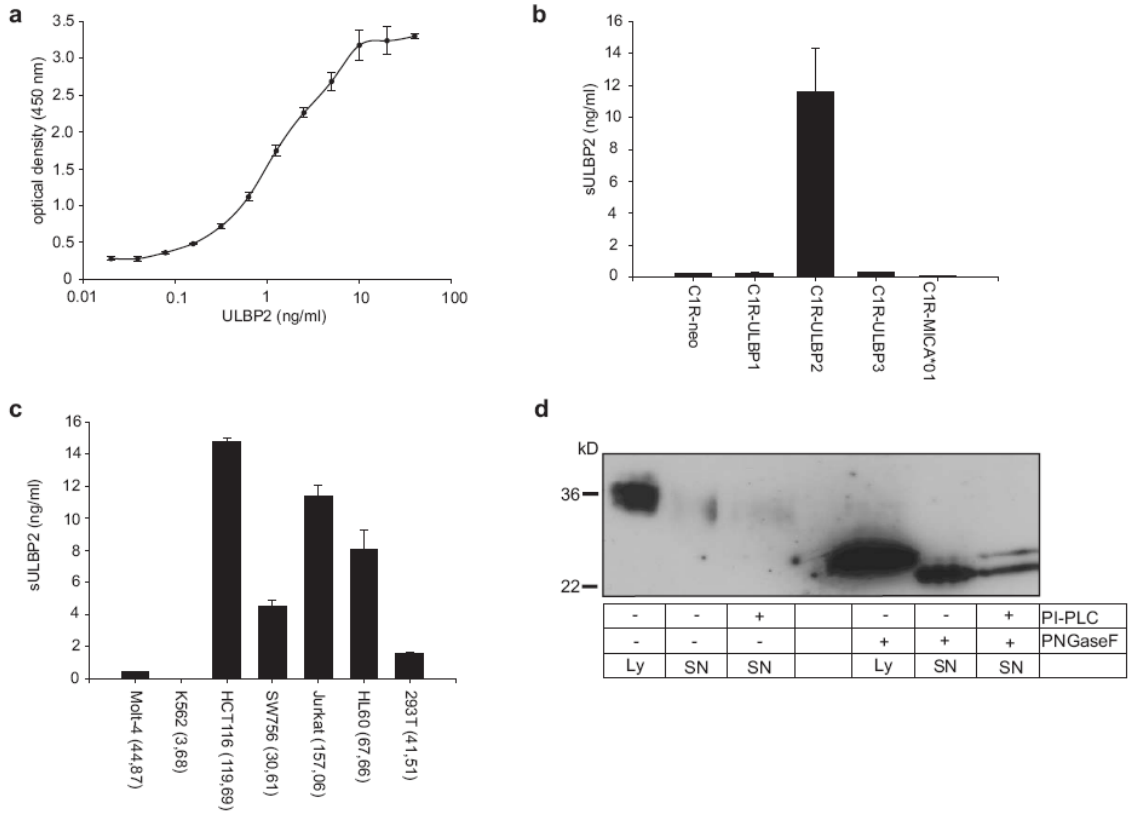

Figure 2.1. Soluble ULBP2 is released by tumour cells. (a) ULBP2 sandwich-ELISA. Serial dilutions of recombinant ULBP2-Fc were analyzed in a sandwich of anti-ULBP2-specific mAb BUMO1 and mAb 165903. **(b)** Supernatants of C1R cells stably transfected with ULBP1, ULBP2, ULBP3, MICA*01, and vector alone were analyzed by ULBP2 ELISA after 16 h culture in fresh medium. ULBP surface expression of the various C1R-ULBP transfectants has previously been shown [8]. **(c)** Supernatants of various cell lines endogenously expressing ULBP2 (SFI of ULBP2 surface expression in brackets) were analyzed by ULBP2 ELISA after 36 h culture in fresh medium. The data shown in a-c are means of triplicates with standard deviations of a representative experiment from a total of three. **(d)** ULBP2 immunoblot of fourfold concentrated supernatants or lysates of C1R-ULBP2 cells. Supernatants were from PI-PLC-treated or untreated C1R-ULBP2 cells. Samples were treated with N-glycanase (PNGase F) before SDS-PAGE where indicated.

Next, we assayed ULBP2 in lysates and supernatants of C1R-ULBP2 cells by immunoblotting and detected molecular species of about 30-35 kDa with a diffuse appearance most likely due to heterogeneous glycosylation. In line with this presumption, pretreatment of the C1R-ULBP2 lysate with PNGase F resulted in a distinct band of lower molecular weight corresponding to the expected size of mature ULBP2 protein (~24 kDa) **(Figure 2.1d)**. Deglycosylated sULBP2 detected in the C1R-ULBP2 supernatant was of even lower apparent molecular weight suggesting enzymatic processing of the membrane-bound protein. ULBP2 is attached to the cell membrane by a GPI-anchor which previously was shown by treatment of ULBP2-transfected cells

with PI-PLC [**17**]. Enzymatic release of several GPI-linked proteins by phospholipases has been reported [**19**]. Therefore we treated C1R-ULBP2 transfectants with PI-PLC to analyze phospholipase-shed ULBP2 molecules. Unexpectedly, PNGase F-treated supernatants of PI-PLC-treated C1R-ULBP2 cells contained an additional band of higher molecular weight corresponding to the ULBP2 species in cell lysates suggesting that the naturally released sULBP2 is not generated by the activities of phospholipases **(Figure 2.1d)**.

ULBP2 molecules are released by metalloproteases

To address an involvement of metalloproteases in the shedding of ULBP2, we treated C1R-ULBP2 cells with metalloprotease inhibitor III (MMPI III) and observed a pronounced reduction of ULBP2 release correlating with increasing concentrations of the inhibitor **(Figure 2.2a)**. Similar data were obtained using the broad metalloprotease inhibitor batimastat (BB94) that has previously been tested in phase I/II trials in cancer patients. To verify that shedding by metalloproteases is not a peculiarity of C1R-ULBP2 transfectants, we treated Jurkat and 293T cells with MMPI III and BB94, respectively, and also found a dose-dependent reduction of ULBP2-levels in the respective supernatants **(Figure 2.2b** and data not shown). Next, we investigated sULBP2 levels in supernatants of C1R-ULBP2 cells that were treated with bacterial phospholipase PI-PLC and detected ~1.3 fold higher sULBP2 concentrations compared to supernatants of mock-treated cells **(Figure 2.2c)**. Whereas physiological ULBP2 shedding was largely inhibited by BB94, there was only a slight reduction of sULBP2 levels in the supernatants of PI-PLC-treated C1R-ULBP2 cells suggesting that BB94 does not affect the activity of phospholipases. Altogether these data demonstrate that ULBP2 is released from tumour cells by metalloproteases, not by phospholipases.

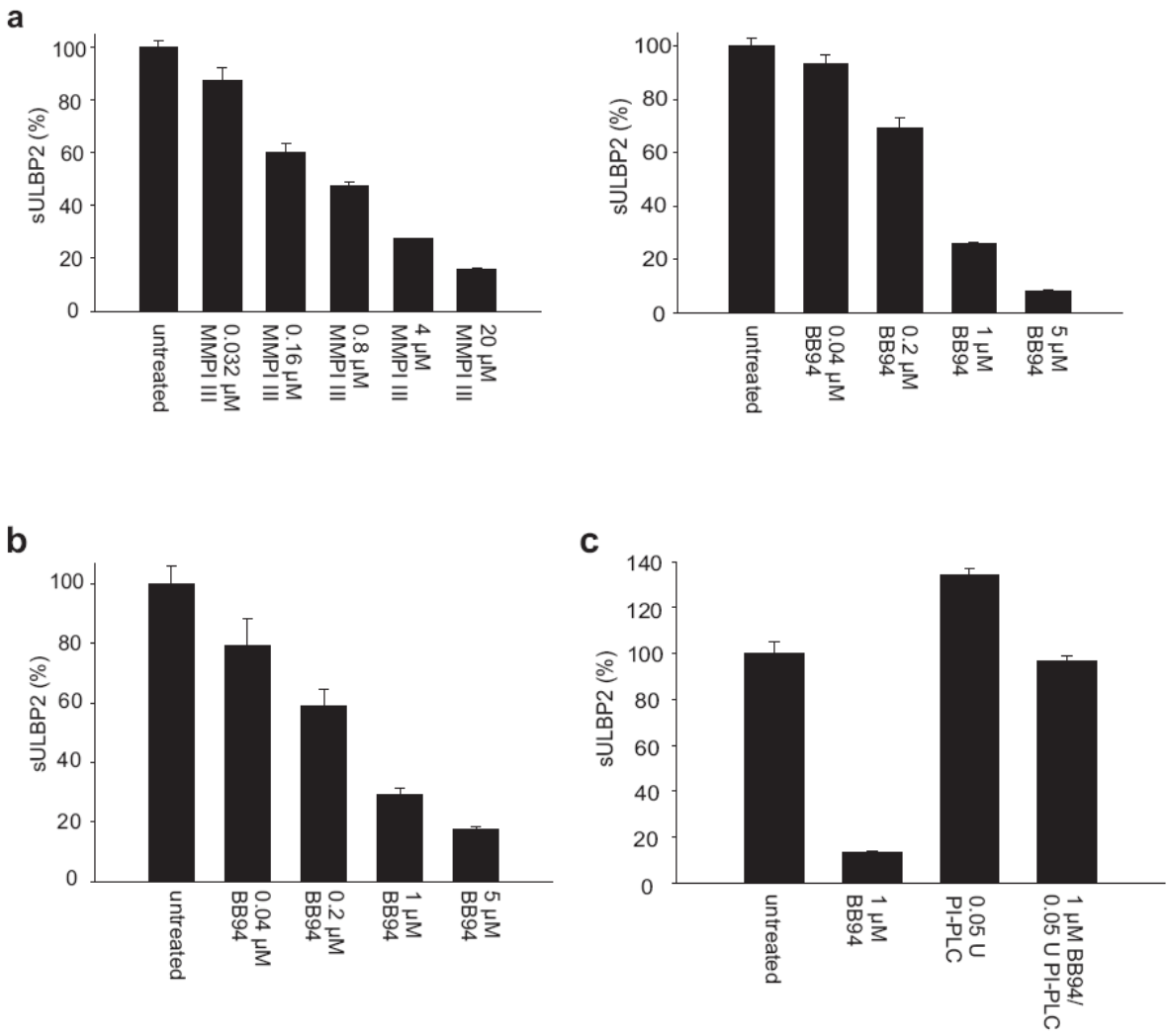

Figure 2.2. ULBP2 is released by metalloproteases. (a) C1R-ULBP2 cells were incubated for 16 hours with MMPI III or BB94 at various concentrations and, subsequently, supernatants analyzed by ULBP2 ELISA. **(b)** Jurkat cells were incubated for 20 hours with BB94 at various concentrations and, subsequently, supernatants analyzed by ULBP2 ELISA. **(c)** Soluble ULBP2 was determined in supernatants of C1R-ULBP2 cells treated with PI-PLC and/or BB94 for 2 hours at 37°C by ULBP2 ELISA. **(a-c)** Concentrations of sULBP2 in supernatants of untreated cells were set as 100%. Data shown are means of triplicates with standard deviations of one representative experiment from a total of three.

Proteolytic shedding of ULBP2 is enhanced by PMA

To further characterize the ULBP2 shedding activity, we investigated the short-term effect of PMA-treatment on ULBP2 release. Whereas most members of the matrix metalloproteinase family (MMP) are released as soluble enzymes and thus are not expected to respond to PMA-mediated activation of Protein Kinase C (PKC), an enhanced proteolytic activity has been reported for members of the membrane-bound "A Disintegrin and Metalloprotease" (ADAM) proteins following PMA-treatment [**20**].

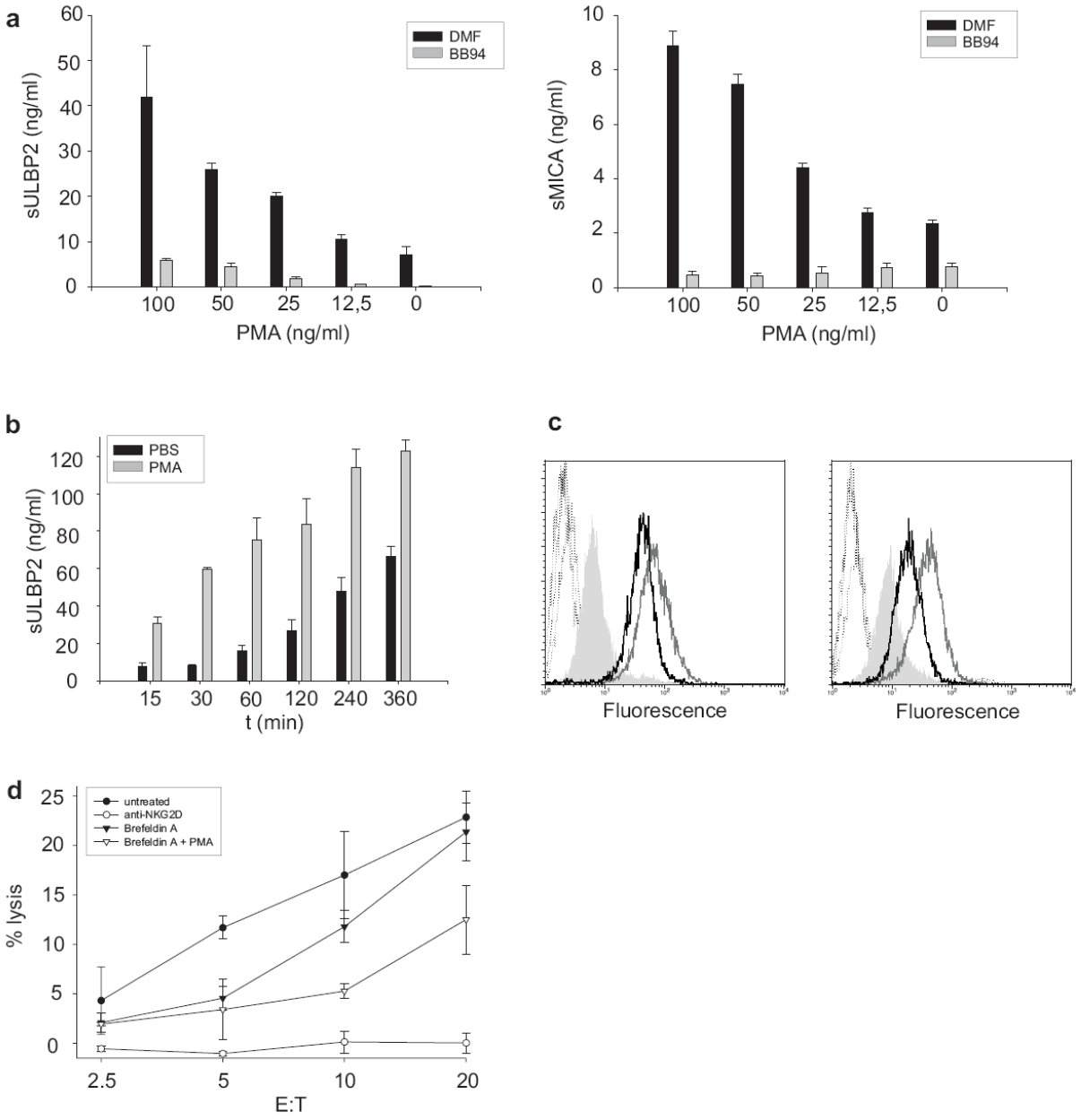

Figure 2.3. PMA stimulates shedding of ULBP2 and MICA. (a) C1R-ULBP2 cells (left) or C1R-MICA*01 cells (right) were pre-incubated with 5µM BB94. After 20 min cells were incubated with various concentrations of PMA for six hours and subsequently supernatants analyzed by ULBP2 ELISA or MICA ELISA, respectively. **(b)** C1R-ULBP2 cells were incubated with or without 100 ng/ml PMA for different times and, subsequently, supernatants analyzed by ULBP2 ELISA. **(a, b)** Data are means of triplicates with standard deviations. **(c)** Flow cytometric analysis of 293T stained with BUMO1 and soluble NKG2D tetramers, respectively. 293T cells were pretreated with Brefeldin A (BFA) and PMA (filled histogram), BFA alone (black line), or untreated (grey line). Dotted lines represent control stainings with irrelevant IgG1 (BUMO1) or MHC class I tetramer. **(d)** Cytotoxicity assay of untreated 293T cells (filled circles) or 293T cells pretreated with BFA (filled triangles) or BFA and PMA (open triangles) using NKL cells. NKG2D-blockade abrogated lysis of 293T cells by NKL completely (open circles).

We detected up to five-fold increased concentrations of sULBP2 in the supernatants of PMA-treated C1R-ULBP2 cells as compared to solvent-treated cells **(Figure 2.3a)**. Enhancement of ULBP2 shedding was dependent on the PMA-concentration and could largely be blocked by addition of BB94. We wondered whether shedding of MICA succumbs to the same mechanisms as ULBP2 shedding. Therefore we also treated C1R-MICA transfectants with PMA and analyzed sMICA levels in the culture supernatants with our previously described MICA sandwich-ELISA [**8**]. It turned out that also MICA shedding is induced by PMA-treatment and inhibited by BB94 **(Figure 2.3b)**. We then investigated the kinetics of PMA-induced ULBP2 shedding and observed that sULBP2 in supernatants of PMA-treated C1R-ULBP2 cells was about fourfold increased as early as 15 min after begin of treatment as compared to mock-treated cells **(Figure 2.3c)**. Conversely, PMA-induced shedding resulted in a decrease of ULBP2 and overall NKG2DL surface levels of Brefeldin A pretreated 293T cells **(Figure 2.3c)**. Reduced NKG2DL surface levels were paralleled by a marked reduction in NKG2D-mediated NK lysis of 293 T cells **(Figure 2.3d)**.

Soluble ULBP2 in sera of patients with malignant diseases

Soluble MICA has been detected in many sera of patients with haematopoietic and epithelial malignancies [**8,12,13,16,21**]. Accordingly, we analyzed sera of patients with leukaemias and gastrointestinal tumours for elevated levels of sULBP2. Four out of 23 sera from leukaemic patients contained substantial levels of sULBP2 (range: 0.09-0.72 ng/ml) including patients with T-NHL, AML and CML **(Figure 2.4a)**. Interestingly, high levels of sULBP2 in a patient with T-NHL correlated with the previously described pronounced ULBP2 surface expression on the respective malignant cells [**8**]. In contrast, 19 sera of patients with gastrointestinal malignancies and 14 of 15 sera from healthy donors contained no detectable sULBP2. Previous studies reported NKG2D-downregulation by cell-bound and soluble MICA [**12,16,21**]. We investigated a similar phenomenon for ULBP2. Upon co-cultivation of the NKG2D expressing cell line NKL with C1R-ULBP2, a pronounced down-regulation of surface NKG2D was observed, but upon co-cultivation with control C1R-neo transfectants **(Figure 2.4b)**. When we treated NKL cells with supernatants of both C1R-neo and C1R-ULBP2 cells, we did not observe NKG2D down-regulation though C1R-ULBP2 supernatants contained sULBP2 at ~100 ng/ml and **(Figure 2.4c)**. We verified that our tumour cell-derived sULBP2 was capable to interact with NKG2D by immobilizing ULBP2 from the supernatants of

C1R-ULBP2 cells on protein A-coated microspheres and staining with fluorochrome-conjugated NKG2D tetramers **(Figure 2.4d)**. Accordingly, pretreating NKL cells with sULBP2 (100ng/ml) for 24 h did not affect cytotoxicity of NKL cells against 293T cells (data not shown).

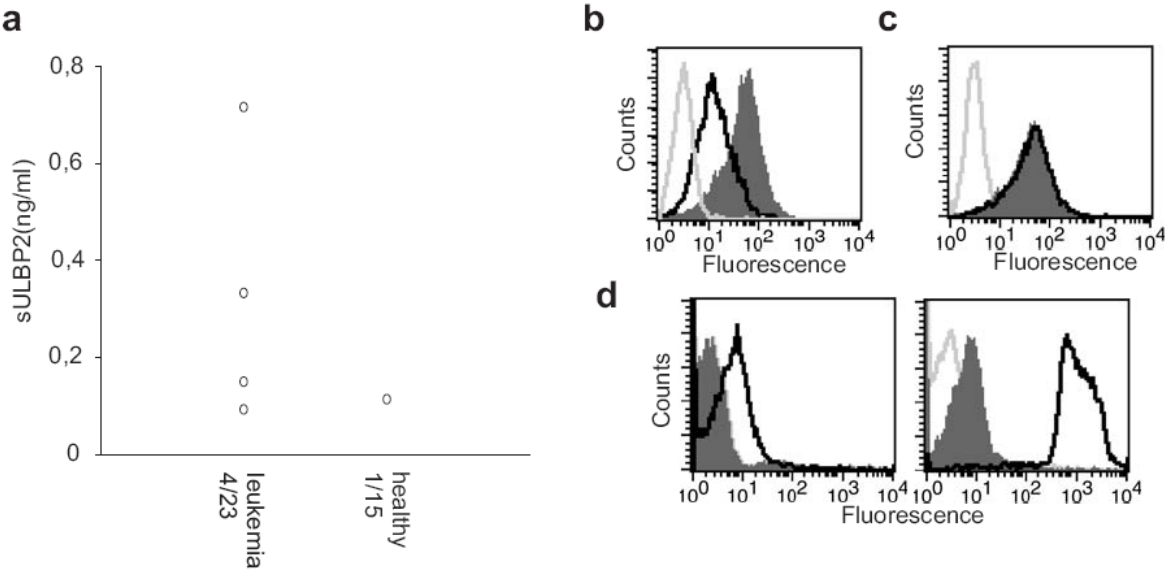

Figure 2.4. Soluble ULBP2 in sera of leukaemic patients. (a) Four of 23 sera from patients with leukaemia and one of 14 sera from healthy donors, but none of 19 sera from patients with gastrointestinal malignancies contained sULBP2 at detectable levels (> 0.05 ng sULBP2/ml serum). Soluble ULBP2-containing sera were from patients UPN1, UPN3, UPN12, and UPN20 with T-NHL, AML, sAML and CML, respectively [8]. Data are means of triplicates. **(b)** NKG2D expression by NKL cells after co-culture with C1R-ULBP2 or C1R-neo cells, respectively. NKG2D surface expression of NKL cells was determined by flow cytometry using mAb 139 after 24 hours incubation with irradiated C1R-neo (filled histograms) or C1R-ULBP2 transfectants (open histograms, dark lines). Isotype control stainings are light lines. **(c)** NKL cells were incubated for 24 h with concentrated supernatants from C1R-neo cells (filled histograms) or from C1R-ULBP2 cells containing 100 ng/ml sULBP2 (open histograms, dark line). Isotype control stainings are light lines. **(d)** Microbeads coated with mAb BUMO1 were incubated with supernatants from C1R-ULBP2 (right) or C1R-neo cells (left), respectively, and stained with NKG2D tetramers (dark lines) or H2-Kd tetramers (light lines). As additional controls, W6/32 coated microbeads were incubated with C1R-neo and C1R-ULBP2 supernatants and stained with NKG2D tetramers (filled histograms).

2.1.5 Discussion

Shedding of the human NKG2DL MICA has recently been described as a novel immune escape mechanism of tumours [**12,13,16,21**]. Here, we investigated tumour cell

shedding of ULBP2 as a representative of GPI-anchored NKG2DL. A previous study reported release of ULBP2 by several tumour cell lines and proposed shedding by phospholipases [**18**]. We however find that ULBP2 shedding is, at least for the most part, executed by metalloproteases and not by phospholipases. Shedding of both MICA and ULBP2 is susceptible to the same set of metallprotease inhibitors and inducible by PMA suggesting the involvement of the same or related metallproteases **(Figure 2.3b** and data not shown). PMA-inducible shedding has been reported for a variety of other cell surface proteins like TNF-α, TGF-α and HB-EGF that are shed by members of the ADAM family of transmembrane metalloproteases, e. g. ADAM17 [**20**]. Current work in our laboratory investigates involvement of ADAMs in the shedding of NKG2DL.

Proteolytic shedding of membrane proteins may either regulate cell surface expression levels and/or promote release of biological active soluble isoforms. For NKG2DL, regulation of cell surface expression is of crucial importance, since NKG2DL surface levels critically determine the susceptibility to NKG2D-stimulated cytolysis [**9,17**]. We demonstrate that PMA-induced shedding of ULBP2 results in markedly reduced ULBP2 surface levels and paralleled by reduced NKG2DL surface densities as well as impaired NKG2D-mediated NK lysis. Further, soluble MICA from sera of tumour patients has also been described to cause a systemic impairment of anti-tumour cytotoxicity by down-regulation of NKG2D on peripheral CD8 T cells and NK cells [**12,16,21**]. With regard to sULBP2, we did not observe down-regulation of NKG2D on NK cells using concentrations of tumour-cell derived soluble ULBP2 that were well above the concentrations in sera of patients with leukaemia. Interestingly, we did not detect sULBP2 in sera of patients with gastrointestinal tumours whereas sMICA was broadly detected in sera of patients with gastrointestinal and other tumours [**12,13,16,21**]. Though this may indicate that ULBP2 is primarily expressed and shed by malignant hematopoietic cells, several tumour cell lines of epithelial origin have also been reported to express ULBP molecules [**17,22**]. However, expression of ULBP by epithelial tumours in vivo has yet to be shown.

In summary, we here report that soluble ULBP2 molecules originate from tumour cells by metalloproteolytic cleavage and are detectable in sera of some patients with leukaemia. Shedding of ULBP2 reduces NKG2DL surface levels and may impair immunogenicity of tumour cells. Further studies have to address the molecular mechanics of ULBP2 cleavage, the relevance of ULBP shedding for tumour immunity,

and the potential of sULBP as novel parameter for diagnosis and/or prognosis in leukaemia.

2.1.6 Acknowledgement

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2.1.7 References

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2.2 TGF-β and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells

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The author of this thesis performed all ULBP2 sandwich ELISAs in **Figure 2.8B**.

2.2.1 Abstract

NKG2D ligands (NKG2DL) are expressed by infected and transformed cells. They transmit danger signals to NKG2D-expressing immune cells leading to lysis of NKG2DL-expressing cells. We here report that the NKG2DL MICA/B and ULBP1-3 are expressed in human brain tumours in vivo, while expression levels are low or undetectable in normal brain. MICA and ULBP2 expression decrease with increasing WHO grade of malignancy, while MICB and ULBP1 are expressed independently of tumour grade. We further delineate two independent mechanisms that can explain these expression patterns: (i) Transforming growth factor-β (TGF-β) is upregulated during malignant progression and selectively down-regulates MICA, ULBP2 and ULBP4 expression while MICB, ULBP1 and ULBP3 are unaffected. (ii) Cleavage of MICA and ULBP2 is reduced by inhibition of metalloproteinases (MP), whereas no changes in expression levels of other NKG2DL were detected. Consequently, NKG2DL-dependent NK cell-mediated lysis is enhanced by depletion of TGF-β or inhibition of metalloproteinases. Thus escape from NKG2D-mediated immune surveillance of malignant gliomas is promoted by the inhibition of MICA and ULBP2 expression via an autocrine TGF-β loop and by metalloproteinase-dependent shedding from the cell surface. These data define MICA and ULBP2, in contrast to other NKG2DL, as particularly important in glioma immune escape and show a differential regulation of

human NKG2DL expression as part of the immunosuppressive properties in human malignant gliomas.

2.2.2 Introduction

NKG2D was identified as an activating receptor on natural killer (NK) cells mediating responses to stress-induced ligands [**1**]. NKG2D exerts additional roles in innate immunity by activating γδ T cells [**2**] and in adaptive immunity by providing costimulatory signals to CD8+ αβ T cells [**3,4**]. Triggering of NKG2D initiates a perforin-mediated cytolytic response against virally infected and tumourigenic cells [**5**]. To accomplish its specific targeting, NKG2D interacts with different MHC class I-homologous ligands. In humans, these are MHC class I-chain related molecules A (MICA) and MICB [**6**], UL16-binding proteins (ULBP)1-3, ULBP4/RAET1E and RAET1G [**7-9**]. Distinct expression patterns indicate that the various NKG2DL are not simply redundant in function [**10**]. With the exception of intestinal epithelia normal cells in adults show absent or low level NKG2DL expression [**11**]. In pathological conditions, however, NKG2DL expression is often up-regulated, as described for epithelial tumours, gliomas and infected cells [**1-3,12-14**]. NKG2DL are induced by genotoxic stress and blocked DNA replication, conditions known to activate a DNA damage response pathway [**15**]. NKG2D is down-regulated by transforming growth factor-β (TGF-β) [**16-18**]. TGF-β production has been associated with the growth and malignant progression of a large variety of tumours including gliomas [**17,19-21**] and interferes with several mechanisms of anti-tumour immune responses [**22**].

Tumour cells may also evade NKG2D-mediated immune surveillance by shedding MIC und ULBP molecules via metalloproteinases (MP) [**23-26**]. This is of particular interest since MP inhibitors are clinically available. Currently there are three known classes of metalloproteinases: MMP (matrix metalloproteinase), ADAM (a disintegrin and metalloproteinase) and ADAM-TS (ADAM with thrombospondin). Glioma cells are known to release high levels of MMP [**27-30**]. The MMP family is comprised of 25 structurally related proteinases either secreted into the extracellular milieu or tethered to the cellular surface [**31**]. MMP expression and activation is increased in almost all human cancers compared with normal tissue [**32**] and they have been implicated in tumour invasion and metastasis due to their ability to degrade the extracellular matrix barrier [**33,34**]. The related ADAMs protein family consists of 40 different members

[**35**]. ADAMs regulate the tumour-related EGFR and ErbB2 signalling pathways [**36,37**] and are overexpressed in a variety of cancers. In gliomas, expression of ADAM 12 is upregulated with increasing grade of malignancy [**38**]. Whereas ADAMs are transmembrane proteins, ADAM-TS are secreted. Currently 19 different ADAM-TS are known, all of which are characterized by a metalloproteinase, a disintegrin and a C-terminal thrombospondin-like domain [**39**]. ADAM-TS4 has been found to be responsible for brevican cleavage in glioma cells and may therefore be critical in mediating invasiveness [**40**]. However, MP do not only clear the path for invading tumour cells, but also regulate the availability of a large variety of cell surface molecules including proteinase inhibitors, adhesion molecules, growth factor binding proteins, cell surface receptors and immunoregulatory proteins [**32,37,39**]. Of note, both TGF-β and MP expression are upregulated with increasing grade of glioma malignancy [**38,41-44**] indicating a prominent role in the malignant properties.

Here we show that gliomas of different grades of malignancy express the NKG2DL MICA, MICB and ULBP1-3 in vivo. In the LNT-229 glioma cell line, they are differentially suppressed by TGF-β at the mRNA level and selectively cleaved from the cell surface by MP. The elucidation of these mechanisms discloses novel avenues for the therapeutic induction of anti-tumour immune responses.

2.2.3 Materials and methods

Human tissue specimens. To study the expression of MICA/B, ULBP1-3, TGF-β_1 and -β_2 in the normal human brain, 18 white matter tissue samples from a normal brain bank were investigated. Further, 20 cases each of gliomas of the WHO grades II (diffuse astrocytoma), III (anaplastic astrocytoma) and IV (glioblastoma) were studied.

Cell lines and transfectants. The human malignant glioma cell line LN-229 was originally provided by Dr. N. de Tribolet (Lausanne, Switzerland) and renamed LNT-229 for clarification (T for Tübingen). The cells were maintained in DMEM supplemented with 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), 10% FCS (Biochrom KG) and penicillin (100 IU/ml)/streptomycin (100 µg/ml) (Gibco). The generation of LNT-229 TGF-$\beta_{1/2}$ siRNA cells has been described **[17]**.

Antibodies and flow cytometry. The following mAbs were used for the assessment of cell surface expression or the blocking of NKG2D, NKG2DL, CD3 and CD56: MAB 139 IgG1 anti-NKG2D and ILU01 polyclonal goat anti-human ULBP2 were from R&D Systems (Wiesbaden, Germany), HIT3a IgG2a anti-CD3-FITC and B159 IgG1 anti-CD56-PE from BD Pharmingen (Heidelberg, Germany), AMO1 IgG1 anti-MICA, BAMO1 IgG1 anti-MICA/B, BAMO3 IgG2a anti-MICA/B, BMO2 IgG1 anti-MICB, AUMO1 IgG1 anti-ULBP1, BUMO1 IgG1 anti-ULBP2, CUMO1 IgM anti-ULBP3 have been published before **[14]** and CUMO3 IgG1 anti-ULBP3 was newly generated. Cells were blocked with 2% BSA and incubated with the specific mAb or matched isotype antibodies (5 μg/ml) for 30 min on ice. Specific binding was detected with PE-conjugated goat anti-mouse IgG (Sigma, Deisenhofen, Germany). Fluorescence was measured in a Becton Dickinson FACScalibur. Specific fluorescence indices (SFI) were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody.

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded samples using the Benchmark system (Ventana, Strasbourg, France). After blocking of endogenous peroxidase, anti-MICA (AMO1, IgG1, 1:25), anti-MICB (BMO2, IgG1, 1:50), anti-ULBP1 (AUMO1, IgG1, 1:100) and anti-ULBP3 (CUMO3, IgG1, 1:25) antibodies were applied. Binding specificity was controlled by IgG1 isotype controls (DakoCytomation, Hamburg, Germany). For visualization, I-View horseradish-peroxidase conjugated streptavidine was applied, followed by diaminobenzidine/H2O2. For ULBP2, samples were immersed in citrate buffer and irradiated in a microwave oven. Endogenous peroxidase was blocked and standard porcine serum was applied to prevent non-specific binding. Polyclonal goat anti-human ULBP2 antibody (clone ILU01, R&D Systems) was followed by biotinylated rabbit anti-goat immunoglobulins (DakoCytomation). Diaminobenzidine was used as chromogen. All sections were counterstained with haematoxylin. Using an Olympus BX50 microscope, two raters quantitated expression levels by the following scale: 0 signifies absence of detectable staining, 1 corresponds to single positive cells in a focal pattern, 2 denotes positive cells in diffuse patterns, 3 indicates up to 20% of positive cells, 4 was allotted when the percentage was between 20% and 50%, 5 was given for more than 50% of positive cells.

Purification of peripheral blood lymphocytes (PBL) and isolation of NK cells. PBL were prepared by density gradient centrifugation (Biocoll, Biochrom KG, Berlin, Germany) and depletion of plastic-adherent monocytic cells. PBL were cultured on irradiated RPMI 8866 feeder cells to obtain polyclonal NK cell populations [**45**].

Real-time PCR. Total RNA was prepared using RNAeasy (Qiagen, Hilden, Germany) and transcribed according to standard protocols. cDNA amplification was monitored using SYBRGreen chemistry on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Primers and conditions for 18S rRNA, MICA/B and ULBP1-3 have been published [**46**]. Primer sequences for ULBP4 were forward: 5´-CTGGCTCAGGGAATTCTTAGG-3´ (573-593), reverse: 5´-CTAGAAGAAGACCAGTGG ATATC-3´ (665-643). Relative induction levels (rI) of NKG2DL were calculated by the formula

$$rI = 2^{-[(\text{threshold cycle NKG2DL - threshold cycle 18S})_{\text{sample}} - (\text{threshold cycle NKG2DL - threshold cycle 18S})_{\text{untreated cells}}]}.$$

Soluble MICA and ULBP2 ELISA. Glioma cell supernatants were analysed in sandwich ELISAs for soluble MICA (sMICA) using anti-MICA mAb AMO1 and BAMO3 or for soluble ULBP2 (sULBP2) using anti-ULBP2 mAb BUMO1 and 165903 (R&D) as previously described [**24,26**].

Inhibition of metalloproteinase activity. The broad spectrum MP inhibitors GM6001 (N-[(2R)-2 hydroxyamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) (Chemicon, Hampshire, UK) and MMP inhibitor III (Merck, Darmstadt, Germany) were dissolved at 50 mM into DMSO and used at 10 µM working concentrations.

Cytotoxicity assay. Cytotoxicity was assessed in 4 h ^{51}Cr release assays in the absence or presence of various mAb. The antibody concentrations for the blocking experiments were 10 µg/ml. NK cells were pretreated with normal human IgG to prevent antibody-dependent cellular cytotoxicity before they were co-incubated for 4 h with 1 x 10^4 ^{51}Cr-labeled target cells per well at various effector:target (E:T) ratios. Spontaneous ^{51}Cr release was determined by incubating the target cells with medium alone. Maximum release was determined by adding NP-40 (2%). The percentage of ^{51}Cr release was

calculated as follows: 100 x ([experimental release – spontaneous release]/[maximum release – spontaneous release]).

Statistics. The experiments shown were repeated at least three times with similar results. Where indicated, analysis of significance was performed using the two-tailed Student's t-test with $P<0.05$ considered significant and $P<0.01$ considered highly significant (Excel, Microsoft, Redmond, WA). For the assessment of in vivo expression levels, the scores for the relative staining intensities were compared between the various tumour entities using the Kruskal-Wallis test.

2.2.4 Results

NKG2DL expression in human gliomas in vivo

We examined gliomas with different WHO grades of malignancy (Kleihues and Cavenee, 2000) for the expression levels of NKG2DL using immunohistochemistry **(Figure 2.5)**. In normal central nervous system tissue specimens white matter showed in general undetectable or very low NKG2DL expression levels on cells exhibiting features of reactive astrocytes. In contrast, WHO grade II astrocytomas displayed an almost uniformly upregulated expression of MICA/B and ULBP1-3 on tumour cells **(Figure 2.5B-F)**. MICA expression levels decreased in gliomas of higher grades of malignancy **(Figure 2.5B)**. In contrast, MICB **(Figure 2.5C)** and ULBP1 **(Figure 2.5D)** showed no significant downregulation. ULBP2 expression was strongest again on WHO grade II astrocytomas whereas expression levels were lower in grade III anaplastic astrocytomas and very low in grade IV **(Figure 2.5E)**. The percentage of ULBP3-positive cells was generally lower than the other NKG2DL in gliomas of all grades. However, the general pattern was similar with ULBP3 expression being very low or even absent on normal brain and upregulated in WHO grade II astrocytomas **(Figure 2.5F)**. ULBP3 expression remained elevated in grade III gliomas and was only slightly decreased in grade IV gliomas. The findings from 18 normal brain specimens and from 20 cases each of grade II, III and IV gliomas are summarized in **Table 2.1**.

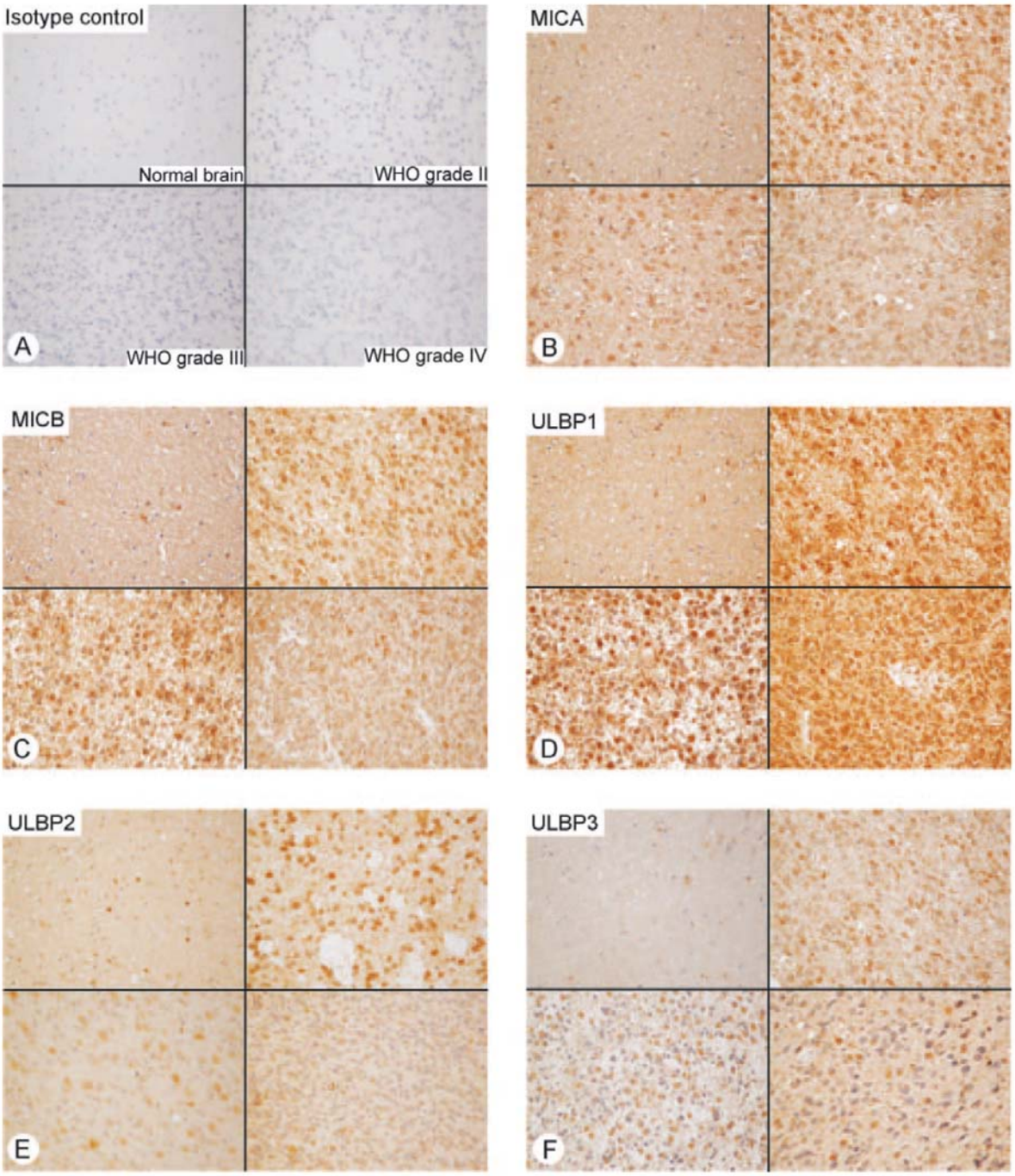

Figure 2.5. NKG2DL expression in human gliomas in vivo. Normal white matter (upper left quadrant in each group of four), WHO grade II astrocytoma (upper right), WHO grade III astrocytoma (lower left) and glioblastoma (WHO grade IV) (lower right) were stained with either isotype control (**A**, representative IgG1 isotype control is depicted) or specific MICA **(B)**, MICB **(C)**, ULBP1 **(D)**, ULBP2 **(E)** or ULBP3 **(F)** antibodies.

Table 2.1. Tumour grade dependence of NKG2DL expression in human gliomas in vivo.

	MICA (mean ± SE)	MICB (mean ± SE)	ULBP1 (mean ± SE)	ULBP2 (mean ± SE)	ULBP3 (mean ± SE)
NB	1.2 ± 0.9	1.6 ± 1.1	1.4 ± 1.0	1.3 ± 0.9	0.8 ± 0.6
II	3.9 ± 0.8	3.7 ± 0.9	3.6 ± 1.0	4.3 ± 0.7	2.4 ± 1.0
III	2.9 ± 0.8	3.3 ± 1.3	3.2 ± 0.9	3.4 ± 1.1	2.4 ± 1.3
IV	2.3 ± 0.8	2.5 ± 1.2	3.4 ± 1.2	1.9 ± 1.0	1.4 ± 0.9
	** *** ** *	* *** ns ns	*** *** ns ns	ns *** ** *	* *** ns *

The relative staining intensities for MICA, MICB, ULBP1, ULBP2 and ULBP3 were rated on a scale ranging from 0 to 5 and compared between white matter tissue samples from a normal brain bank (NB, n=18) and gliomas of WHO grades II, III and IV (n=20 each; not significant^{ns}; P<0.05*; P<0.01*; P<0.001*).

TGF-β-mediated suppression of MICA and ULBP2 inhibits NKG2D-mediated tumour cell lysis

We and others have previously reported an inhibitory effect of TGF-β on MICA expression [**17,24,25**] and a positive effect of TGF-β on MMP expression and activity [**47**]. To further characterize the role of endogenous glioma cell-derived TGF-β in the regulation of NKG2DL expression, we used LNT-229 cells transfected with siRNA targeted against TGF-β_1 and TGF-β_2 [**17**]. Real-time PCR revealed a marked induction of MICA, ULBP2 and ULBP4 mRNA transcripts in TGF-$\beta_{1/2}$ siRNA cells whereas the levels of MICB, ULBP1 and ULBP3 remained unaltered **(Figure 2.6A)**. To correlate the changes in NKG2DL mRNA expression induced by TGF-β with changes at the protein level, we performed flow cytometry using antibodies specific for the respective NKG2DL. Consistent with the mRNA data, MICA and ULBP2 expression on the cell surface of TGF-$\beta_{1/2}$ siRNA cells was increased by factors of 5.0±1.0 and 2.4±0.2 whereas no change was observed with MICB, ULBP1 or ULBP3. One representative out of three experiments is shown in **Figure 2.6B**. For ULBP4, there is currently no specific antibody available. The re-exposure of TGF-$\beta_{1/2}$ siRNA cells to exogenous TGF-β repressed the induction of MICA, ULBP2 and ULBP4 mRNA **(Figure 2.6C)** and also decreased the cell surface expression of MICA and ULBP2 by 40±8% and 20±1% **(Figure 2.6D;** n=3).

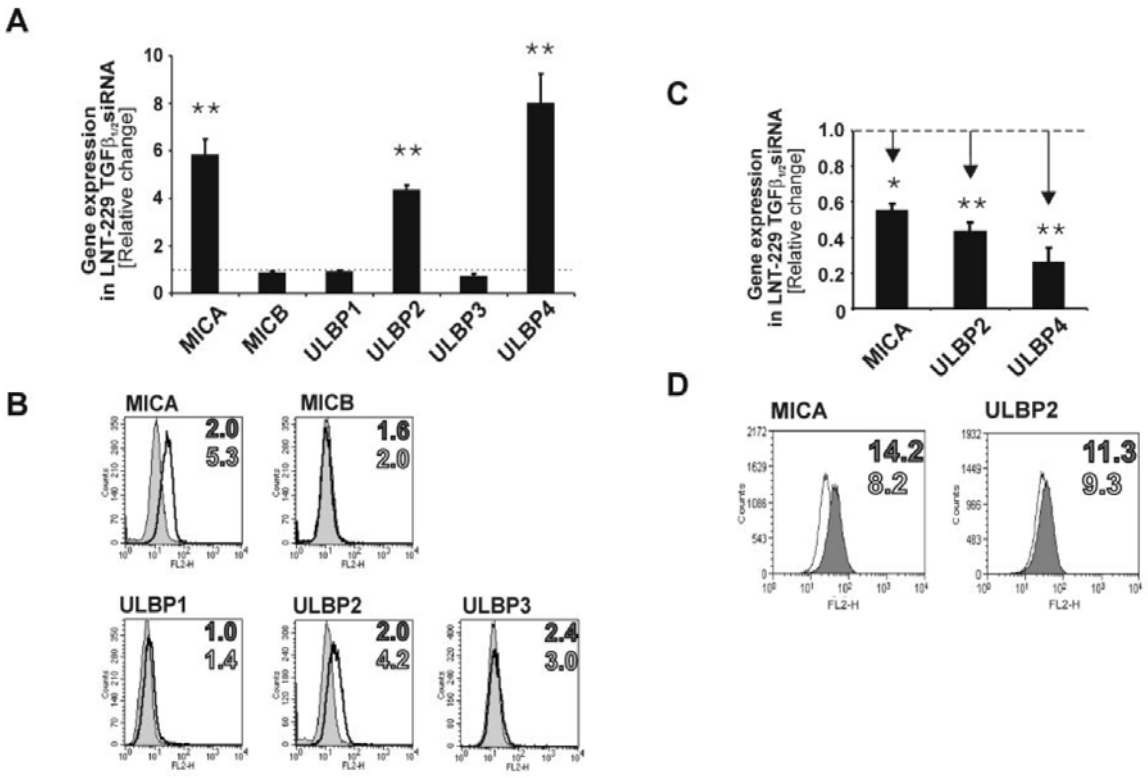

Figure 2.6. NKG2DL mRNA expression and protein levels at the cell surface: modulation by siRNA targeting TGF-$\beta_{1/2}$. (A) MICA, MICB, ULBP1, 2, 3 and 4 mRNA expression was assessed in control or TGF-$\beta_{1/2}$ siRNA cells by real-time PCR. **(B)** MICA, MICB, ULBP1, 2 and 3 expression at the cell surface of control (closed profiles) and TGF-$\beta_{1/2}$ siRNA cells (open profiles) was assessed by flow cytometry. **(C)** TGF-$\beta_{1/2}$ siRNA cells were untreated or treated with TGF-β_2 (10 ng/ml) for 48 h and assessed for MICA, ULBP2 and ULBP4 mRNA expression. **(D)** TGF-$\beta_{1/2}$ siRNA cells, untreated (closed profiles) or treated (open profiles) with TGF-β_2 (10 ng/ml) for 7d, were analyzed accordingly. Data in **A** and **C** are expressed as the relative gene expression compared with control transfectants in **A** or untreated TGF-$\beta_{1/2}$ siRNA cells in **C** set to 1. Data represent mean values ± SEM from three independent experiments. In **B** and **D** SFI values are indicated in the upper right corner and are representative of three independent experiments.

The changes in the expression levels of MICA, ULBP2 and ULBP4 resulted in an increase in NK cell-mediated lysis of TGF-$\beta_{1/2}$ siRNA cells **(Figure 2.7)**. The increased glioma cell lysis by polyclonal NK cells was partially blocked by either neutralizing MICA or ULBP2 mAb. The incubation with both mAbs was equivalent to a blocking NKG2D mAb.

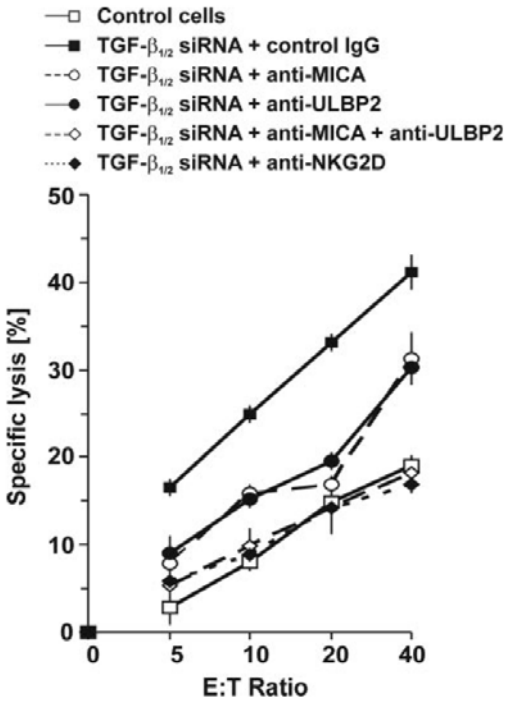

Figure 2.7. MICA and ULBP2 enhance NK cell lysis in TGF-$\beta_{1/2}$ siRNA cells. Control or TGF-β1/2 siRNA cells untreated or treated with anti-MICA (AUMO1), anti-ULBP2 (BUMO1), with both antibodies or anti-NKG2D (MAB 139) (10 μg/ml) were used as target cells in a standard 4 h ^{51}Cr release assay using polyclonal NK cells as effectors. Data are expressed as specific lysis at different E:T ratios. One representative experiment of three is shown. Each data point was generated from triplicates. Coefficients of variation ranged below 5% for each data point.

Selective and TGF-β-independent shedding of MICA and ULBP2 by metalloproteinases inhibits NK cell-mediated lysis.

As shown previously [**47**], TGF-β promotes MP expression (especially MMP-2 and -9) in glioma cells. Since NKG2DL surface expression can be modulated on a post-translational level by proteolytic cleavage [**23-26,48,49**], we asked whether shedding of sMICA and sULBP2 was reduced in TGF-$\beta_{1/2}$ siRNA cells [**17**], thus contributing to the enhanced surface expression of MICA and ULBP2 on these cells. With control cells, inhibition of MP activity by exposure to the broad spectrum MP inhibitors GM6001 and MMP inhibitor III enhanced the expression levels of MICA 2.4-fold (±0.1) and of ULBP2 1.4-fold (±0.1) at the cell surface whereas no significant changes were detected in MICB, ULBP1 and 3 expression **(Figure 2.8A,** upper panel, **Table 2.2** and data not shown; n=3). A transcriptional effect of the inhibitor on NKG2DL mRNA levels was ruled out by real-time PCR (data not shown). Parallel experiments were also performed to study the effects of MP inhibition in the TGF-$\beta_{1/2}$ siRNA cells. Despite the reduced

levels of MMP-2 and -9 activity in TGF-$\beta_{1/2}$ siRNA cells, MP inhibition superinduced the expression levels of MICA 1.3-fold (±0.03) and of ULBP2 1.5-fold (±0.05) **(**Figure 2.8**A,** lower panel, **Table 2.2;** n=3). Further, immunoblotting of cell culture supernatant showed that the basal level of sMICA released by TGF-$\beta_{1/2}$ siRNA cells was markedly enhanced compared with the control cells paralleling higher surface expression (data not shown). While these findings demonstrate that the cleavage of NKG2DL is not mediated by TGF-β-dependent proteases, the release of sMICA and sULBP2 from LNT-229 control and TGF-$\beta_{1/2}$ siRNA cells in cell culture supernatant could nevertheless be reduced by ~ 50% for sMICA and ~ 80% for sULBP2 when GM6001 was added, as shown by MICA- and ULBP2-specific ELISA **(Figure 2.8B)**.

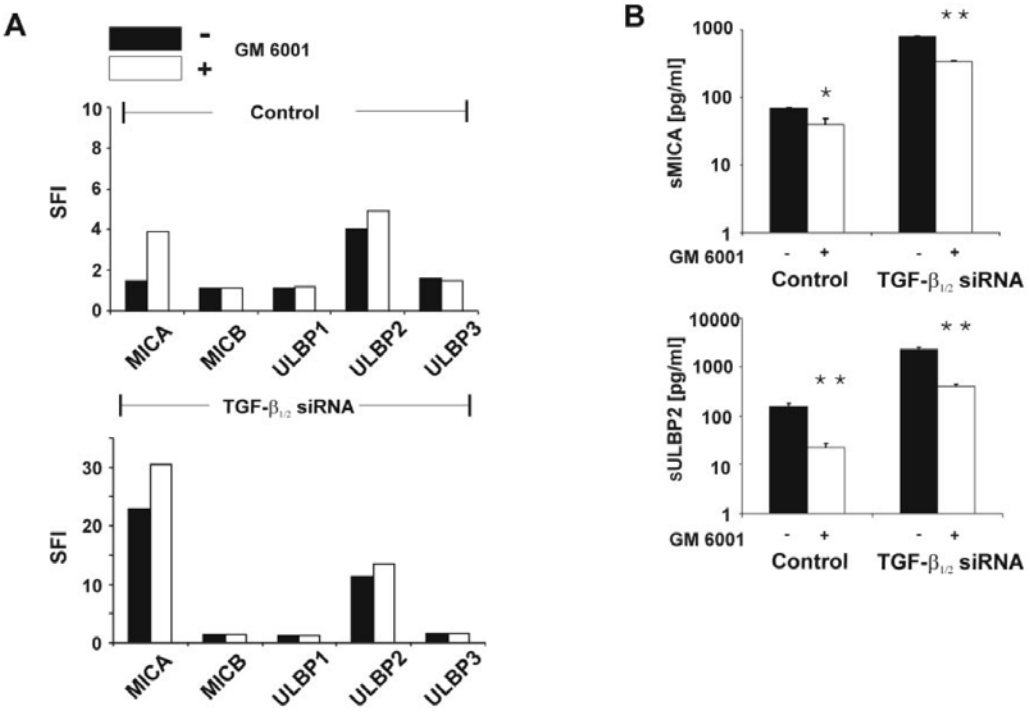

Figure 2.8. MP inhibition modulates NKG2DL expression in glioma cells. (A) MICA/B and ULBP1-3 levels at the cell surface of LNT-229 control or TGF-$\beta_{1/2}$ siRNA cells exposed to GM6001 for 48 h were determined by flow cytometry. One representative experiment of three is shown. SFI represent specific fluorescence indices and are calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with isotype-matched control antibody. **(B)** Levels of sMICA (upper panel) and sULBP2 (lower panel) were analyzed by ELISA, using supernatants from LNT-229 control or TGF-$\beta_{1/2}$ siRNA cells exposed to GM6001 (10 μM) for 48 h (n=3). Note the logarithmic scale.

Table 2.2. MP inhibition enhances MICA and ULBP2 surface expression.

	MICA	MICB	ULBP1	ULBP2	ULBP3
	Mean fold induction ± SEM				
Control transfectants	2.4 ± 0.12	1.2 ± 0.12	0.9 ± 0.09	1.4 ± 0.09	1.0 ± 0.03
TGF-β1/2 siRNA cells	1.3 ± 0.03 *	0.9 ± 0.06	1.1 ± 0.03	1.5 ± 0.05 ns	1.0 ± 0.05

MICA/B and ULBP1-3 levels at the cell surface of LNT-229 control transfectants or TGF-$\beta_{1/2}$ siRNA cells exposed to GM6001 for 48 h were determined by flow cytometry. Mean fold inductions of three independent experiments and SEM are shown. While there is no effect for MICB, ULBP1 and 3, MICA and ULBP2 are consistently upregulated. The degree of induction on control transfectants and TGF-$\beta_{1/2}$ siRNA cells was similarly for ULBP2 (P=1.0, ns) but significantly stronger on controls for MICA (P<0.05).

Next we assessed the functional consequences of reduced NKG2DL shedding in a ^{51}Cr release assay using target glioma cells pre-treated with GM6001 for 48 h. As expected, MP inhibition enhanced the NK cell-dependent killing of LNT-229 control glioma cells.

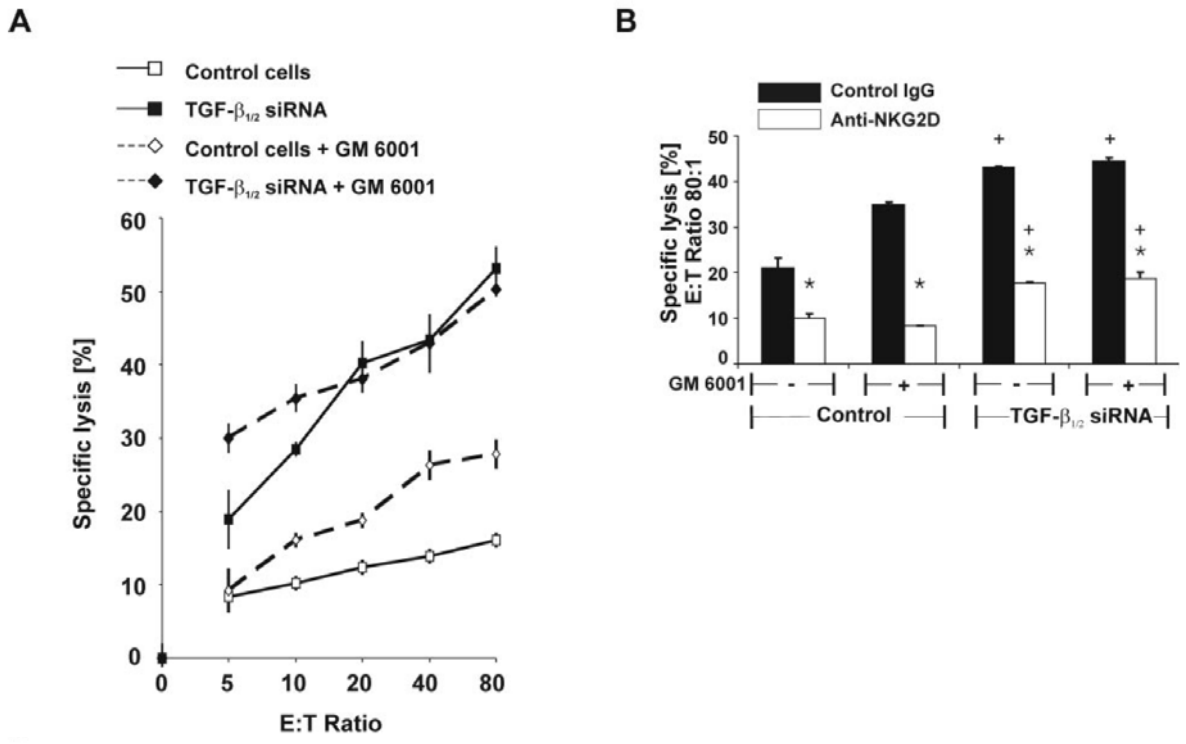

Figure 2.9. MP inhibition enhances the immunogenicity of glioma cells.

(A) LNT-229 control or TGF-$\beta_{1/2}$ siRNA cells were untreated or exposed to GM6001 (10 µM) for 48 h before they were subjected to a standard 4 h ^{51}Cr release assay using polyclonal NK cells as effectors. Data are expressed as specific lysis at different E:T ratios. **(B)** Glioma cells untreated or treated with GM6001 (10 µM) for 48 h were used as targets in a standard 4 h ^{51}Cr release assay in the absence or presence of control IgG or anti-NKG2D (10 µg/ml each), using polyclonal NK cells as effectors. Data are expressed as specific lysis at an E:T ratio of 80. One representative experiment of three is shown. Each data point was generated from triplicates. Coefficients of variation ranged below 4% for each data point.

Further, the high constitutive NKG2DL expression in TGF-$\beta_{1/2}$ siRNA cells resulted in massive target cell lysis that was superinduced by GM6001 when E:T ratios were low and lysis conditions presumably suboptimal **(Figure 2.9A)**. The co-exposure of LNT-229 control or TGF-$\beta_{1/2}$ siRNA cells to blocking NKG2D mAb attenuated the lysis of glioma cell targets **(Figure 2.9B)**.

Other activating molecules that may also be regulated by MP activity do not seem to play a decisive role in this context since the enhanced lysis of GM6001-pretreated glioma cells was nullified by NKG2D mAb. However, a difference in susceptibility towards NK cell-mediated lysis between LNT-229 control and TGF-$\beta_{1/2}$ siRNA cells persisted in the presence of NKG2D mAb, suggesting that other mechanisms interfering with NK-cell mediated killing are also regulated by TGF-β [**16**].

2.2.5 Discussion

NKG2DL are expressed almost de novo in gliomas in vivo and may thus label tumour cells for recognition by NKG2D-expressing immune effector cells **(Figure 2.5, Table 2.1)**. However, MICA and ULBP2 expression levels are downregulated close to or to baseline levels in grade IV tumours. This implies that the immunogenicity of gliomas decreases with increasing grade of malignancy, possibly due to a selection process favouring the survival of less immunogenic glioma cells. Surprisingly, elevated MICB and ULBP1 expression levels do not change during the course of malignant progression and ULBP3 was only slightly downregulated in grade IV gliomas. Due to the lack of a suitable antibody, ULBP4 expression could not be assessed in vivo. Interestingly, all examined NKG2DL were expressed at low levels also on single astrocytes in normal brain, with scores ranging from 0.8 for ULBP3 to 1.6 for MICB on our scale. These astrocytes showed reactive changes which were considered as morphological alterations induced by mortal agony. Since NKG2DL are known to be stress-inducible, their expression on astrocytes undergoing reactive changes might be due to terminal hypoxia-induced stress in these patients.

It remains unclear whether high cellular NKG2DL expression confers any survival advantage during the development of gliomas. Most likely, the blood-brain barrier that becomes disrupted in high-grade gliomas but is still intact in lower grade astrocytomas

[**50**] will hamper immune cell infiltration into the brain and thus prevent the NKG2D-mediated elimination of low grade astrocytomas.

Our study identifies two largely independent mechanisms which can explain the different NKG2DL staining patterns in malignant gliomas and their role in escaping NKG2D-mediated anti-tumour immunity. The association between increasing grade of malignancy and elevated expression of immunosuppressive molecules such as TGF-β has already been documented [**17,41-43**]. The mechanisms by which TGF-β undermines anti-tumour immune surveillance [**22,51**] might involve effects on co-stimulatory signals using NKG2D as the target molecule [**17,18**]. Our previous studies had indicated that there is an activation potential for immune cells when NKG2DL are highly expressed on glioma cells [**3**]. However, TGF-β counteracts these mechanisms by enhancing the expression of the inhibitory receptor CD94/NKG2A and by downregulating the activating receptor NKG2D on CD8+ T and NK cells [**3,16,52**].

Here we show that TGF-β additionally down-regulates the transcription of the human NKG2DL MICA, ULBP2 and ULBP4 **(Figure 2.6)**. Interestingly, TGF-β does not interfere with MICB, ULBP1 and ULBP3 mRNA and cell surface expression **(Figure 2.6)**, suggesting that the yet uncharacterized promoters may not respond to TGF-β dependent transcription factors such as SMADs or that their mRNAs are selectively stabilized. Blocking experiments revealed additive functions of MICA and ULBP2 in triggering NKG2D, indicating that NKG2DL at the surface of transformed cells may contribute to anti-tumour immune responses in an additive manner **(Figure 2.7)**. Indeed, the effect of blocking both MICA and ULBP2 equalled that of blocking the receptor. These observations suggest a prominent role for the NKG2DL MICA and ULBP2 in glioma immune surveillance while the other ligands may exert their immune stimulatory functions under different conditions. By promoting the reduction of NKG2DL on the cell surface, tumour cells may efficiently escape innate immune recognition by reducing an induced-self danger signal [**53,54**]. Collectively, these observations confirm that TGF-β is central to the malignant progression of glial tumours and a principle target for the treatment of gliomas [**55**]. Anti-TGF-β therapies may therefore not only relieve the immune dysfunction in human glioblastoma patients, but also act on the tumour cells, restoring MICA, ULBP2 and ULBP4 expression to the levels required for an effective anti-glioma immune response.

Furthermore, TGF-β dependent MP are essential for the migratory and pro-invasive phenotype of glioma cells [**17**]. A direct targeting of MP would also appear to be

justified, not only to impede glioma cell migration and invasion, but also to reduce the proteolytic shedding of NKG2DL. Yet again, MP inhibition by the broad spectrum inhibitors GM 6001 or MMP inhibitor III selectively enhanced the expression of MICA and ULBP2 on the surface of LNT-229 cells but not of other NKG2DL **(Figure 2.8A)**. In parallel, the levels of sMICA in glioma cell supernatant were reduced by ~50% **(Figure 2.8B)** while mRNA levels remained unchanged (data not shown). The fact that MP inhibition predominantly enhanced the cell surface expression of MICA and ULBP2 **(Figure 2.8A)** may be due to the MP equipment of LNT-229 cells and to the low expression level of MICB in these cells. A complete prevention of MICA shedding was not achieved, implicating the involvement of proteases that are unaffected by the inhibitors used. Both GM6001 and MMP inhibitor III inhibit MMP-1, MMP-2 and MMP-3 with GM6001 acting on MMP-8 and MMP-9 as well, while MMP inhibitor III further blocks MMP-7 and MMP-13. Effects of these inhibitors on ADAMs or ADAM-TS are likely, but have not been investigated.

Of note, the relative reduction in sMICA and sULBP2 levels upon treatment with an MP inhibitor did not differ between LNT-229 control and TGF-$\beta_{1/2}$ siRNA cells **(Figure 2.8B)**. Since we have previously shown that down-regulation of TGF-$\beta_{1/2}$ by siRNA results in a striking reduction in MMP-2 and -9 expression and activity, the modulation of NKG2DL surface expression must depend on different MP [**17**].

We also show that the enhanced NKG2DL expression upon exposure to MP inhibitors translates into an increased immunogenicity of glioma cells **(Figure 2.9A, B)**. Of note, at high E:T ratios, a reduction of NKG2DL shedding could not enhance the lysis TGF-$\beta_{1/2}$ siRNA cells any further implicating that the levels of NKG2DL expression achieved in these cells may already have been sufficient to fully activate the NKG2D pathway. Still, the inhibition of MP is a promising option for TGF-β-independent tumours and may also lead to synergy with anti-TGF-β strategies.

Taken together, our in vitro data implicate that the TGF-β-mediated transcriptional repression of MICA and ULBP2 together with the MP-mediated selective shedding of these NKG2DL may be a major factor contributing to the immune escape of higher grade gliomas. This suggested mechanism is strongly supported by the immunohistochemical finding of tumour-specific MICA and ULBP2 becoming downregulated with increasing grade of malignancy. The virtually complete blocking of NKG2D-mediated NK cell lysis of glioma cells by the combination of anti-MICA and anti-ULBP2 antibodies **(Figure 2.7)** strongly suggests that further NKG2DL only play a

subordinate role in glioma biology. The two mechanisms outlined here are particularly attractive since clinically applicable concepts for the inhibition of TGF-β [**56**] and of MP have already been developed and could thus be applied here in a novel context.

2.2.6 Acknowledgement

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2.3 Tumour-associated MICA is shed by ADAM proteases

This chapter has been submitted for publication by the following authors:

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The author of this thesis performed all experiments except the determination of the c-terminus of sMICA in **Figure 2.10A, B** (Dennis Göhlsdorf, Toni Weinschenk), the generation of the MICA mutants (Dennis Göhlsdorf) and the generation of the pSuper-ADAM10 construct (Friederike Gieseke).

2.3.1 Abstract

The immunoreceptor NKG2D promotes immunosurveillance of malignant cells and protects the host from tumour initiation by activating NK cells and costimulating CD8 T cells. NKG2D-mediated recognition of malignant cells by cytotoxic lymphocytes is enabled through the tumour-associated expression of NKG2D ligands (NKG2DL) resulting from cellular stress. Shedding of NKG2DL is thought to constitute a major countermechanism of tumour cells to subvert NKG2D-mediated immunosurveillance. Here, we report that the prototypical NKG2DL MICA is released by proteolytic cleavage in the stalk of the MICA ectodomain where deletions, but not alanine substitutions, impede MICA shedding. Small compound-mediated stimulation and inhibition of MICA shedding adduced characteristics that indicated an involvement of 'a disintegrin and metalloproteinase' (ADAM) family members. Accordingly, silencing of the related ADAM10 and ADAM17 proteases inhibited MICA shedding by tumour cells. Collectively, our data demonstrate that ADAM10 and ADAM17 are critically involved in the tumour-associated proteolytic release of soluble MICA. Hence, therapeutic blockade of ADAM10 and ADAM17 appears promising for cancer treatment by targeting both growth and immune escape of tumours.

2.3.2 Introduction

In recent years, the NKG2D/NKG2DL-system has emerged as a novel tumour immunosurveillance mechanism [**1-3**]. NKG2D is a homodimeric, C-type lectin-like receptor expressed by virtually all human NK cells, CD8 αβ T cells and γδ T cells and, in association with the adaptor protein DAP10, transduces activating signals which stimulate cytotoxicity and cytokine secretion [**4,5**]. Human NKG2DL belong to the families of MHC-encoded MIC molecules (MICA, MICB) and non-MHC-encoded UL16-binding proteins (ULBP) (ULBP1-4, RAET1G, RAET1L) [**5,6**], and typically are not expressed by healthy tissue but rather are induced subsequently to harmful events such as cellular stress or viral infection [**5-8**]. In tumour cells, MICA and other NKG2DL are up-regulated by genotoxic stress dependent on the activity of the DNA damage-detecting protein kinase ATM (ataxia teleangiectasia mutated) [**1**]. Genotoxic stress often occurs in precancerous lesions and many established tumours and, accordingly, MIC molecules are broadly expressed on epithelial tumours and on some haematopoietic malignancies [**1,9-11**] suggesting that they act as 'immuno-alerters' of malignant transformation. Studies in mice demonstrated a potent stimulatory role of NKG2D in tumour immunity: NK and CD8 T cells readily eliminated tumour cells ectopically expressing NKG2DL in a NKG2D-dependent manner, thereby even inducing tumour immunity against the parental tumour cells [**12-14**]. Further, NKG2D also protects mice from tumour initiation [**15,16**].

In established MICA-expressing human tumours, NKG2D-mediated immunosurveillance is antagonized by the release of soluble MICA (sMICA) resulting in a reduction of the overall surface density of NKG2DL on tumours, a systemic NKG2D down-regulation on NK cells and CD8 T cells, and the expansion of immunosuppressive intra-tumoural CD4+NKG2D+ T cells [**17-20**]. Elevated levels of sMICA have been reported for sera of cancer patients with a broad variety of different malignancies [**10,11,18,20,21**].

Previously, we reported that release of sMICA from tumour cells is impaired by metalloprotease inhibitors pointing to an involvement of members of the families of matrix metalloproteases (MMPs) and ADAMs [**20**]. Whereas MMPs are largely implicated in destruction of extracellular matrix, many ADAMs are membrane-tethered proteases that shed transmembranous proteins including cytokines, growth factors and cell-adhesion molecules from the cell surface [**22-24**]. In particular, ADAM10 and

ADAM17 play an eminent role in the shedding of ectodomains. Their catalytic domains are highly related, and, consequently, they share some of their substrates such as amyloid precursor protein (APP) and TNF [**22,24,25**]. They are frequently overexpressed in tumours and are thought to play key roles in different steps of tumourigenesis [**24,26**].

Based on our observations that shedding of MIC molecules is blocked by metalloprotease inhibitors, we set out to characterize the proteolytic activities resulting in MICA release. We anticipated that molecular characterization of the responsible protease(s) may identify new promising targets in the quest for more efficacious immunotherapies of cancer.

2.3.3 Materials and Methods

Transfectants. 293T and HeLa were stably transfected with MICA*01 cDNA using FuGene HD reagent (Roche), C1R with cDNA of MICA mutants by electroporation as described [**20**]. MICA mutants were generated from full-length MICA*01 cDNA (all in RSV.5neo) using Quikchange-Kit (Stratagene, La Jolla, CA) and the following oligonucleotides:

1D 5′-GGAAAGTGCTGGTGCTTCAGACATTCCATGTTTCTG-3′

5′- CAGAAACATGGAATGTCTGAAGCACCAGCACTTTCC-3′

2D 5′-CTCTGCCCTCTGGGTGGCAGACGTTCCATG-3′

5′-CATGGAATGTCTGCCACCCAGAGGGCAGAG-3′

3D 5′-CTGCCCTCTGGGGCTGTTGCTGCTGC -3′

5′-GCAGCAGCAACAGCCCCAGAGGGCAG -3′

1M 5′-CTGCCCTCTGGGAAAGCGGCGGCGGCTCAGAGTCATTGGCAG-3′

5′-CTGCCAATGACTCTGAGCCGCCGCCGCTTTCCCAGAGGGCAG-3′

2M 5′-GGGAAAGTGCTGGTGCTTGCGGCTCATTGGCAGACGTTCCAT-3′

5′-ATGGAATGTCTGCCAATGAGCCGCAAGCACCAGCACTTTCCC-3′

3M 5′-GTGCTGGTGCTTCAGAGTGCTGCGCAGACGTTCCATGTTTCTGC-3′

5′-GCAGAAACATGGAATGTCTGCGCAGCACTCTGAAGCACCAGCAC-3′

4M 5′-GTGCTTCAGAGTCATTGGGCGGCATTCCATGTTTCTGCTG-3′,

5′-CAGCAGAAACATGGAATGCCGCCCAATGACTCTGAAGCAC-3′

5M 5′-CAGAGTCATTGGCAGACAGCCGCTGTTTCTGCTGTTGCTGCTG-3′

5′-CAGCAGCAACAGCAGAAACAGCGGCTGTCTGCCAATGACTCTG-3′

6M 5′-GGCAGACATTCCATGCTGCTGCTGTTGCTGCTGC-3′

5′-GCAGCAGCAACAGCAGCAGCATGGAATGTCTGCC-3′

C1R-MICA transfectants were grown in RPMI 1640 with 1.8 mg G418/ml, and 293T-MICA*01 and HeLa-MICA*01 cells in IMDM with 1.5 mg G418/ml. For proliferation analysis, 293T-MICA cells were labeled with 5 μM CFSE 48 h after transfection, washed, and cultured for four additional days before flow cytometric analysis.

Reagents. PE-labelled human NKG2D tetramers, anti-MICA mAb AMO1, and anti-MICA/B mAb BAMO1 and BAMO3 were previously described [**8**]. Antibodies specific for human ADAM10 (mAb 163003) and human ADAM17 (mAb 111633) were from R&D systems (Minneapolis, MN). HRP-goat anti-mouse Ig, HRP-streptavidin, and PE-goat anti-mouse Ig were from Jackson ImmunoResearch Laboratories (West Grove, PA), HRP-goat anti-mouse IgG2a from Southern Biotechnology Associates (Birmingham, AL). MMPI III, TIMP2 and BIM I were from Merck (Darmstadt, Germany), BB94 was provided by Klaus Maskos, Max-Planck-Institute for Biochemistry, Martinsried, Germany. BB94 was dissolved in dimethylformamide and added in a 1/200 volume to cultures. ADAM-specific metalloprotease inhibitors GW280264 and GI254023 were previously described [**27**]. PMA was from Cell Signaling (Danvers, MA) and CFSE from Sigma (St. Louis, MO).

Flow cytometry. Cells were incubated with indicated antibodies at 10 μg/ml and then, after washing, with PE-goat anti-mouse Ig (1:200) as secondary reagent or, alternatively, with PE-labeled sNKG2D tetramers at 10 μg/ml. Samples were analyzed on a FACScan (BD Bioscience, San Jose, CA). Specific fluorescence intensities (SFI) were calculated by subtracting the mean fluorescence intensity (MFI) of the isotype control from the MFI of the specific antibody.

ELISA. Sandwich ELISA for sMICA based on mAb AMO1 and BAMO3 has been previously described [**10**]. Results are shown as means with SD of triplicates.

Immunoblot analysis. Samples were separated by 10% SDS-PAGE. Gels were blotted to Hybond-ECL membranes (Amersham, Little Chalfont, UK), blocked with TBS containing 5% non-fat dried milk, and then analyzed with BAMO1 followed by a goat anti-mouse HRP-conjugate or with HRP-conjugated streptavidin, and developed with Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

Determination of carboxyterminus of sMICA. Supernatants from C1R-MICA were concentrated, dialyzed, and treated with peptide:N-Glycanase F (New England Biolabs, Beverly, MA) according to manufacturer's instructions. Deglycosylated sMICA was immunprecipitated with mAb BAMO3, separated on a 12.5 % SDS-PAGE and stained with Coomassie Blue. Soluble MICA was excised from the gel, digested with V8 protease (Roche) in the presence of H2O18, and resulting fragments analyzed by MALDI-MS.

RNA silencing. For transient knockdown of human ADAM10, oligonucleotide 5´-ACAGTGCAGTCCAAGTCAA-3´ and its reverse complement, separated by a 9-nt hairpin spacer (ttcaagaga), were inserted into pSuper-puro [**28**]. 293T-MICA cells were transfected with pSuper construct using FuGene HD. For direct delivery of siRNA, 293T-MICA and HeLa-MICA cells were transfected with ADAM17 and/or ADAM10 ON-TARGETplus SMARTpools using DharmaFECT1 (Dharmacon).

ADAM17: 5'-GAAGAACACGUGUAAAUUAUU-3'
5'-GCACAAAGAAUUAUGGUAAUU-3'
5'-UAUGGGAACUCUUGGAUUAUU-3'
5'-GGAAAUAUGUCAUGUAUCCUU-3'

ADAM10: 5'-CAUCUGACCCUAAACCAAAUU-3'
5'-CAAGGGAAGGAAUAUGUAAUU-3'
5'-GAACUAUGGGUCUCAUGUAUU-3'
5'-CGAGAGAGUUAUCAAAUGGUU-3'

As a control ON-TARGETplus siCONTROL non-targeting pool was used.

Real-time RT-PCR. Total RNA was isolated using TriZol (Invitrogen, Karlsruhe, Germany) followed by DNAse I (Promega, Madison, WI) digestion and reverse transcription using M-MLV Reverse Transcriptase (Promega). Resulting cDNA was amplified using ADAM10, ADAM17, MICA and 18S rRNA specific primers using SYBRGreen chemistry on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The ΔCT method was used for relative quantifications. Oligonucleotide sequences (forward, reverse) were for 18S rRNA: 5'-CGGCTACCACATCCAAGGAA-3', 5'-GCTGGAATTACCGCGGCT-3'; MICA: 5'-CCTTGGCCATGAACGTCAGG-3', 5'-CCTCTGAGGCCTCRCTGCG-3'; ADAM10: 5'-CTGGCCAACCTATTTGTGGAA-3', 5'-GACCTTGACTTGGACTGCACTG-3'; ADAM17: 5'-GAAGGCCAGGAGGCGATTA-3', 5'-CGGGCACTCACTGCTATTACC-3'.

2.3.4 Results

Efficiency of MICA shedding depends on the length of the MICA stalk

Our previous work indicated that tumour cell-derived sMICA comprises the entire MICA ectodomain and is derived from membrane-bound MICA by metalloproteolytic cleavage [**20**]. To further characterize the generation of tumour cell-derived sMICA, carboxytermini of sMICA purified from supernatants of C1R-MICA cells were determined by MALDI-MS after proteolytic digestion in presence of H2O18 **(Figure 2.10A)**. Carboxytermini of sMICA mapped to the stalk region (Ser 274 to Ala 291) connecting the MICA α3 domain and the transmembrane domain **(Figure 2.10B)** supporting the notion that sMICA is generated from transmembranous MICA by proteolytic cleavage atop of the plasma membrane. To further characterize MICA cleavage, MICA mutants with deletions or alanine substitutions of the MICA stalk were generated **(Figure 2.10B)** and stably transfected into C1R cells. All MICA mutants with alanine substitutions were expressed on the cell surface at nearly the same level as wildtype MICA. In contrast, only surface expression of mutant 1D was at wildtype levels, whereas surface expression of mutant 2D was markedly reduced and mutant 3D barely detectable **(Figure 2.10C** and data not shown). Similar results were obtained when mutants were analyzed for NKG2D binding using sNKG2D (soluble NKG2D) tetramers indicating that MICA mutants expressed at the cell surface were not conformationally altered **(Figure 2.10C)**. Subsequently, we analyzed supernatants of

transfected C1R cells for sMICA using our previously established MICA ELISA [**10**] **(Figure 2.10D)**. Whereas alanine substitutions in the MICA stalk did not substantially affect MICA shedding, sMICA levels in supernatants of C1R cells expressing mutants 1D and 2D were strongly reduced. These results demonstrate that sMICA is generated by proteolytic cleavage in the stalk region and that the respective protease(s) do(es) not recognize a specific sequence motif within the stalk, but rather is (are) sensitive to shortenings of the stalk.

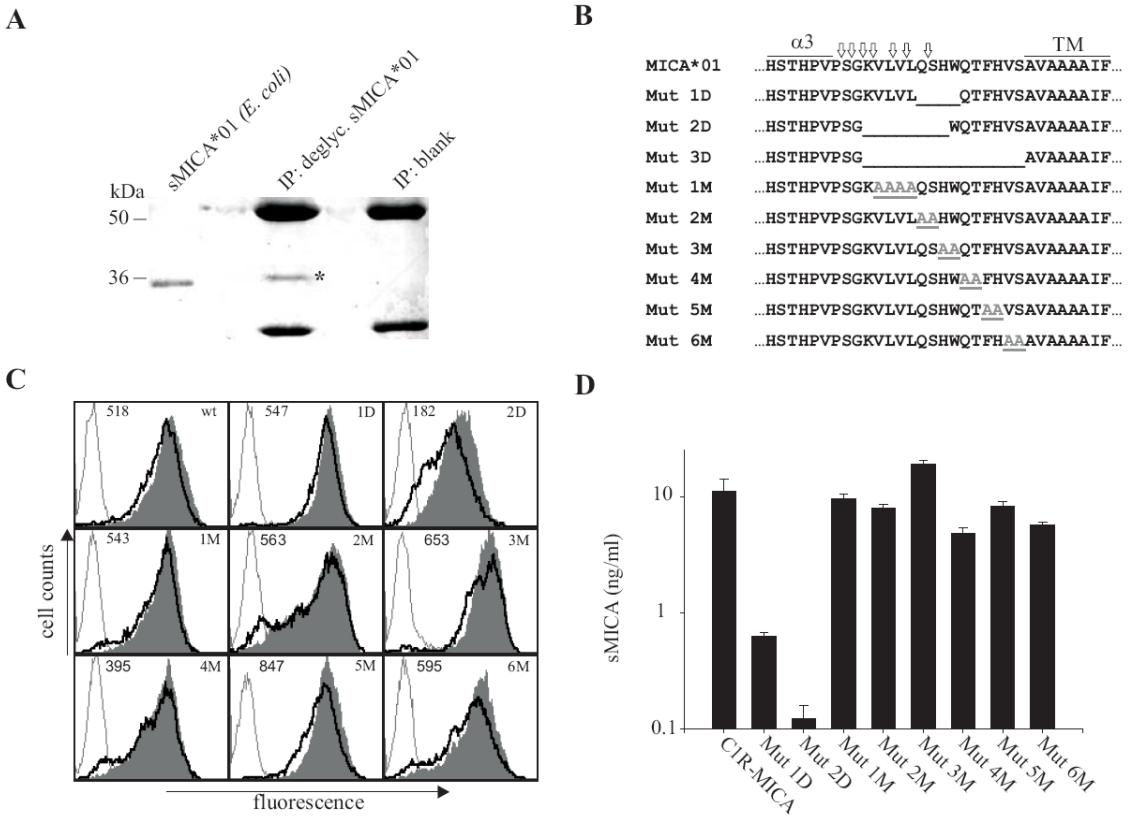

Figure 2.10. MICA cleavage in the stalk region is dependent on length, but not sequence of the stalk. (A) Tumour cell-shed sMICA (asterisk, middle lane) from N-glycanase-treated supernatants of C1R-MICA*04 cells was immunoprecipitated with mAb BAMO3 for mass spectrometric analysis. E. coli-produced sMICA*01 (left lane) and a mock-immunoprecipitation (right lane) are controls. **(B)** Sequence of the MICA stalk connecting the α3 and the transmembrane domain (TM) where vertical arrows indicate carboxytermini of shed sMICA*04 as determined by mass spectrometry (upper line). Lower lines depict mutant MICA sequences with deletions (1D to 3D) or alanine substitutions (1M to 6M), respectively, transfected into C1R cells. **(C)** MICA mutants on stably transfected C1R cells were stained with anti-MICA mAb AMO1 (filled histograms), isotype control IgG1 (grey lines), or sNKG2D tetramers (solid lines). SFI for AMO1 stainings are given. **(D)** Soluble MICA in supernatants of C1R cells stably expressing MICA mutants was determined after 16 h of cultivation using a sMICA-specific sandwich ELISA.

MICA is shed from the cell surface by an ADAM-like proteolytic activity

Next, we attempted to define the cellular compartment where MICA shedding occurs. Hence, cell surface proteins of 293T-MICA cells were biotinylated and, subsequently, both cell lysates and supernatants analyzed for biotinylated MICA at different time points. Importantly, biotinylated MICA was found not only in cell lysates, but also in supernatants, indicating that sMICA is shed from the cell surface **(Figure 2.11)**.

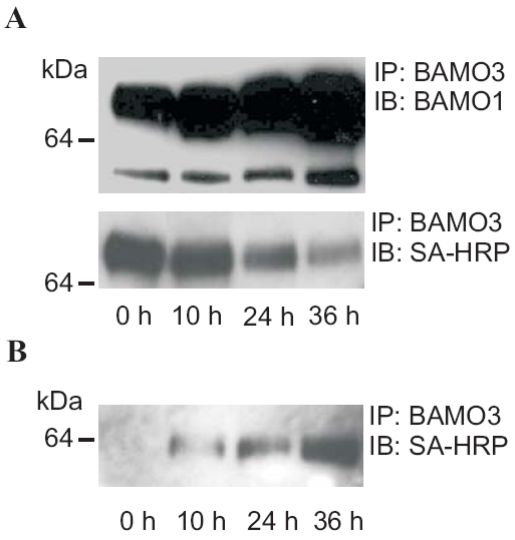

Figure 2.11. MICA is shed from the cell surface. (A,B) 293T-MICA were surface-biotinylated and cultivated for up to 36 h before MIC molecules in cell lysates **(A)** or culture supernatants **(B)** were immunoprecipitated by mAb BAMO3. Biotinylated MICA or total MICA molecules were then detected with streptavidin-HRP (SA-HRP) and anti-MIC mAb BAMO1, respectively.

Next, we evaluated several broad range metalloprotease inhibitors for their ability to inhibit MICA shedding. Matrix metalloprotease inhibitor III (MMPI III) strongly suppressed MICA shedding by C1R-MICA without affecting cell viability **(Figure 2.12A)**. Similar results were obtained for the inhibitors BB94, MMPI II, GM6001 **(**[**29**] and data not shown). In contrast, TIMP2 (tissue inhibitor of metalloproteases-2), a natural and selective inhibitor of MMPs which does not inhibit ADAMs [**30**], did not affect MICA shedding **(Figure 2.12B)**. Previously, we had shown that MICA shedding is stimulated by PMA (phorbol 12-myristate 13-acetate), a characteristic feature of ADAM17-mediated shedding [**31**]. Since PMA is a potent activator of protein kinase C (PKC), we investigated the impact of the PKC-inhibitor Bisindolylmaleimide I (BIM I) on PMA-stimulated MICA shedding. To this aim, we treated C1R-MICA with PMA and BIM I, and analyzed supernatants for sMICA levels **(Figure 2.12C)** and monitored cell surface MICA by flow cytometry **(Figure 2.12D)**. In fact, PMA-stimulated

shedding was inhibited by BIM I in a dose-dependent manner and, at highest BIM I concentrations, reduced to levels of constitutive MICA shedding, whereas BIM I had no effect on MICA shedding of control-treated C1R-MICA cells.

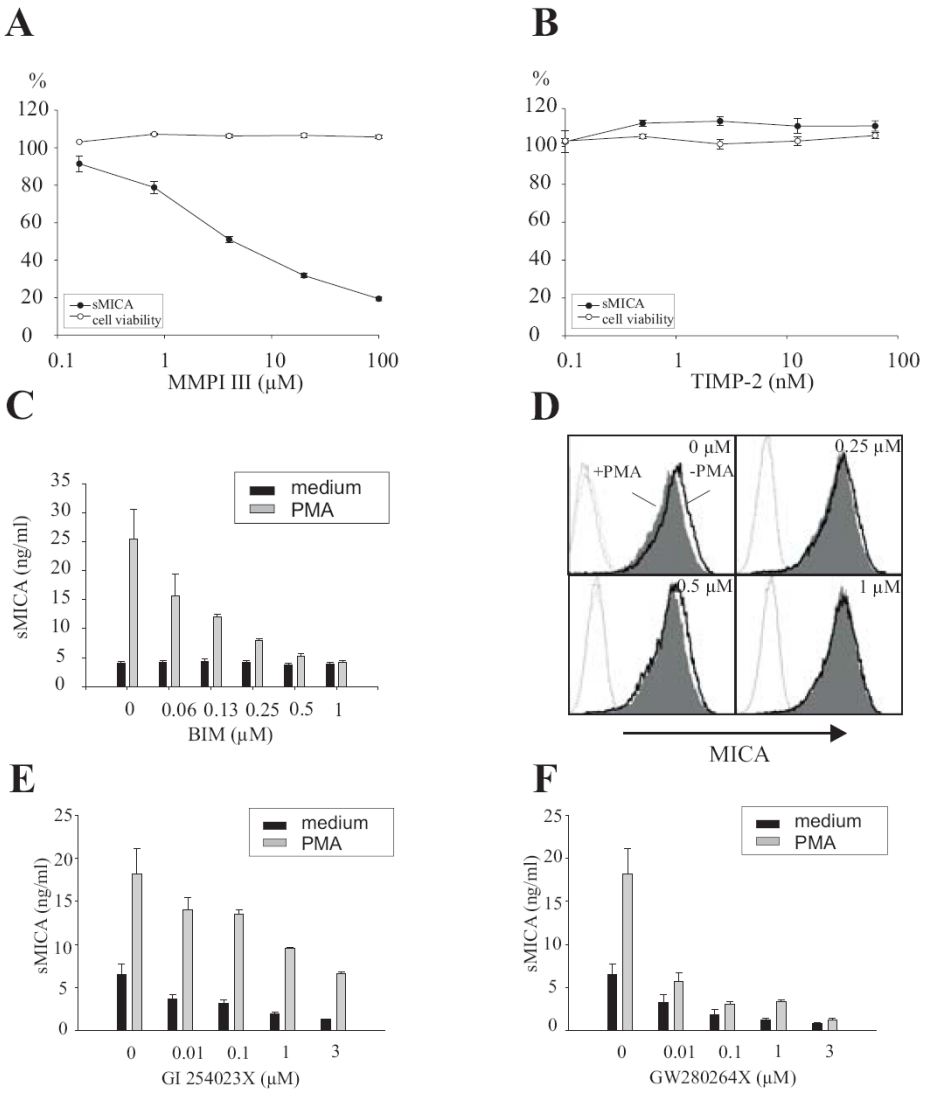

Figure 2.12. Chemical inhibition of MICA shedding reveals characteristics of ADAM-dependent proteolysis. (A, B) MICA shedding by C1R-MICA is repressed by the broad metalloprotease inhibitor MMPI III in a concentration-dependent manner **(A)**, but not by the natural MMP-specific inhibitor TIMP2 **(B)**. Cellular viability was monitored by propidium iodide staining. **(C)** PMA-stimulated shedding of MICA by C1R-MICA was efficiently blocked by pre-incubation with the PKC inhibitor BIM I. **(D)** PMA-stimulated MICA shedding and the BIM I-mediated inhibition is mirrored by alterations of cell surface MICA levels on C1R-MICA. C1R-MICA pre-treated with various concentrations of BIM I were cultured with (grey histograms) or without PMA (open histograms) and stained with mAb AMO1. **(E, F)** PMA-induced MICA shedding by C1R-MICA was potently inhibited by the ADAM-protease inhibitor GW280264X **(F)**, but only inefficiently by the selective ADAM-protease inhibitor GI254023X, whereas both substances equally inhibited constitutive MICA shedding. Levels of sMICA were determined by sMICA-ELISA and PMA was always used at 100 ng/ml, except in **(D)** (50 ng/ml).

Since our results pointed to the involvement of ADAMs in MICA shedding, we studied the effect of ADAM-specific hydroxamate-based metalloprotease inhibitors on MICA shedding. The inhibitor GW280264X reportedly blocks proteolytic activity of both ADAM10 and ADAM17 with comparable efficiency, whereas GI254023X is about 100-fold more effective in blocking ADAM10 than ADAM17 [**27**]. PMA-stimulated MICA shedding of C1R-MICA cells was inhibited by GW280264X in a dose-dependent manner **(Figure 2.12F)**. In contrast, GI254023X inhibited constitutive shedding, but only marginally affected PMA-stimulated shedding **(Figure 2.12E)**. In all experiments, MICA shedding was efficiently blocked by the broad range metalloprotease inhibitor BB94 (data not shown). Collectively, these data suggested that MICA shedding is governed by one or several membrane-bound proteases with ADAM-like activities such as ADAM10 and ADAM17.

ADAM10 and ADAM17 mediate MICA shedding

To directly address an involvement of ADAM10 and ADAM17 in MICA shedding, we transiently silenced expression of ADAM10 and/or ADAM17 in 293T-MICA and HeLa-MICA transfectants by RNA interference. First, we suppressed ADAM10 by transfecting a plasmid expressing an ADAM10 siRNA (pSuper-ADAM10) in 293T-MICA. Down-regulation of ADAM10 was detected by real-time PCR, immunoblotting and FACS in pSuper-ADAM10-transfected, but not in control-transfected cells **(Figure 2.13)**. Concomitantly to ADAM10 suppression, constitutive MICA shedding by 293T-MICA cells was strongly reduced as detected by MICA ELISA **(Figure 2.13D)**. Noteworthy, levels of MICA transcripts and MICA surface expression remained unaltered **(Figure 2.13)**. To assess whether reduced sMICA levels may be due to an impaired proliferation as a consequence of ADAM10 knockdown, pSuper-transfected cells were labeled with CFSE and analyzed after four days of culture **(Figure 2.13E)**. No difference in proliferation was observed between controls and ADAM10-knockdown cells.

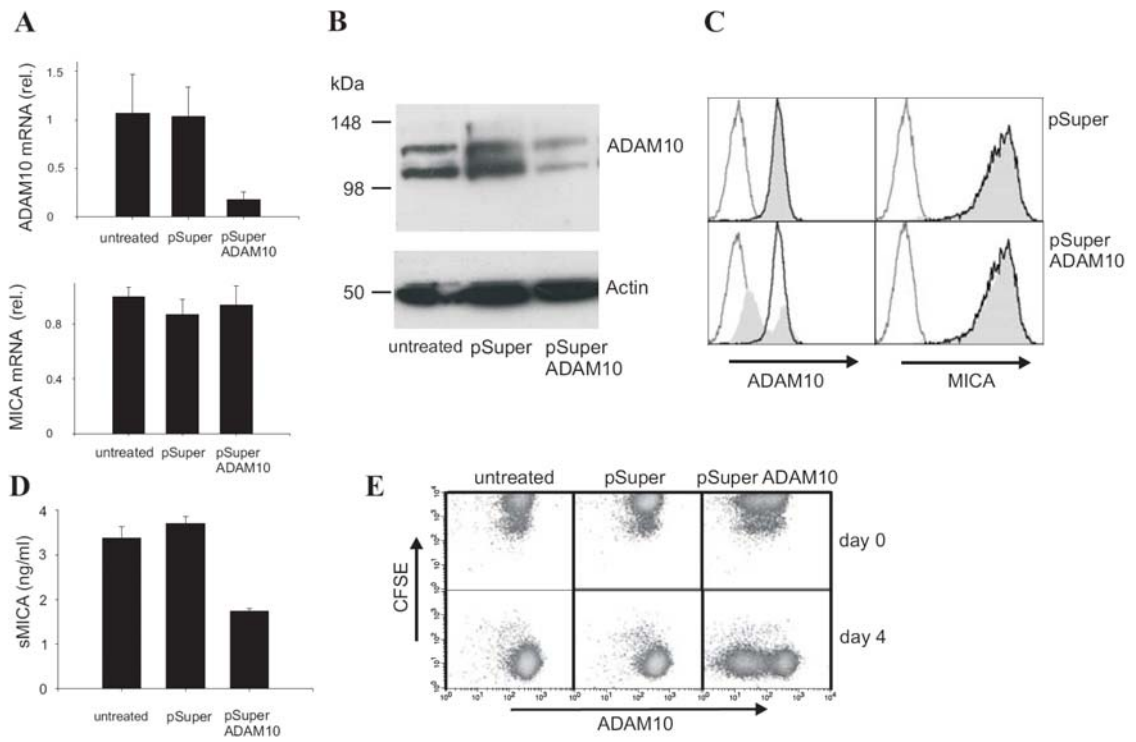

Figure 2.13. ADAM10 silencing impairs constitutive MICA shedding. (A-C) ADAM10 siRNA-expression in 293T-MICA down-regulates ADAM10 transcripts **(A)**, total cellular ADAM10 **(B)**, and cell surface ADAM10 **(C)**. Relative copy numbers of ADAM10 and MICA transcripts in 293T-MICA 48 h after transfection with pSuper or pSuper-ADAM10 and untreated 293T-MICA were determined by real-time PCR after normalization with 18S rRNA **(A)**. Total cellular ADAM10 protein was determined in lysates of 293T-MICA 72 hour after transfection and untreated 293T-MICA cells by immunoblotting **(B)**. Surface expression of MICA or ADAM10 (grey histograms) was determined 72 h after transfection. Stainings of untreated 293T-MICA (thick lines) and with isotype controls (thin lines) are overlayed **(C)**. **(D)** Soluble MICA released from 293T-MICA transfected with pSuper or pSuper-ADAM10 was determined in medium used for culture from 72 h to 75 h post-transfection and compared to 3 h culture supernatants of untreated 293-MICA. **(E)** Transient ADAM10 knock-down did not result in altered proliferation of 293T-MICA. 293T-MICA were transfected with pSuper plasmids, labelled with CFSE, and analysed four days later for surface ADAM10 and CFSE intensity.

In a second approach, we transfected 293T-MICA cells with pools of siRNA targeting ADAM10 and/or ADAM17, respectively, to further analyze an involvement of ADAM10 and ADAM17 in MICA shedding. Successful siRNA-mediated suppression of ADAM expression was again confirmed by real-time PCR and flow cytometry **(Figure 2.14)**.

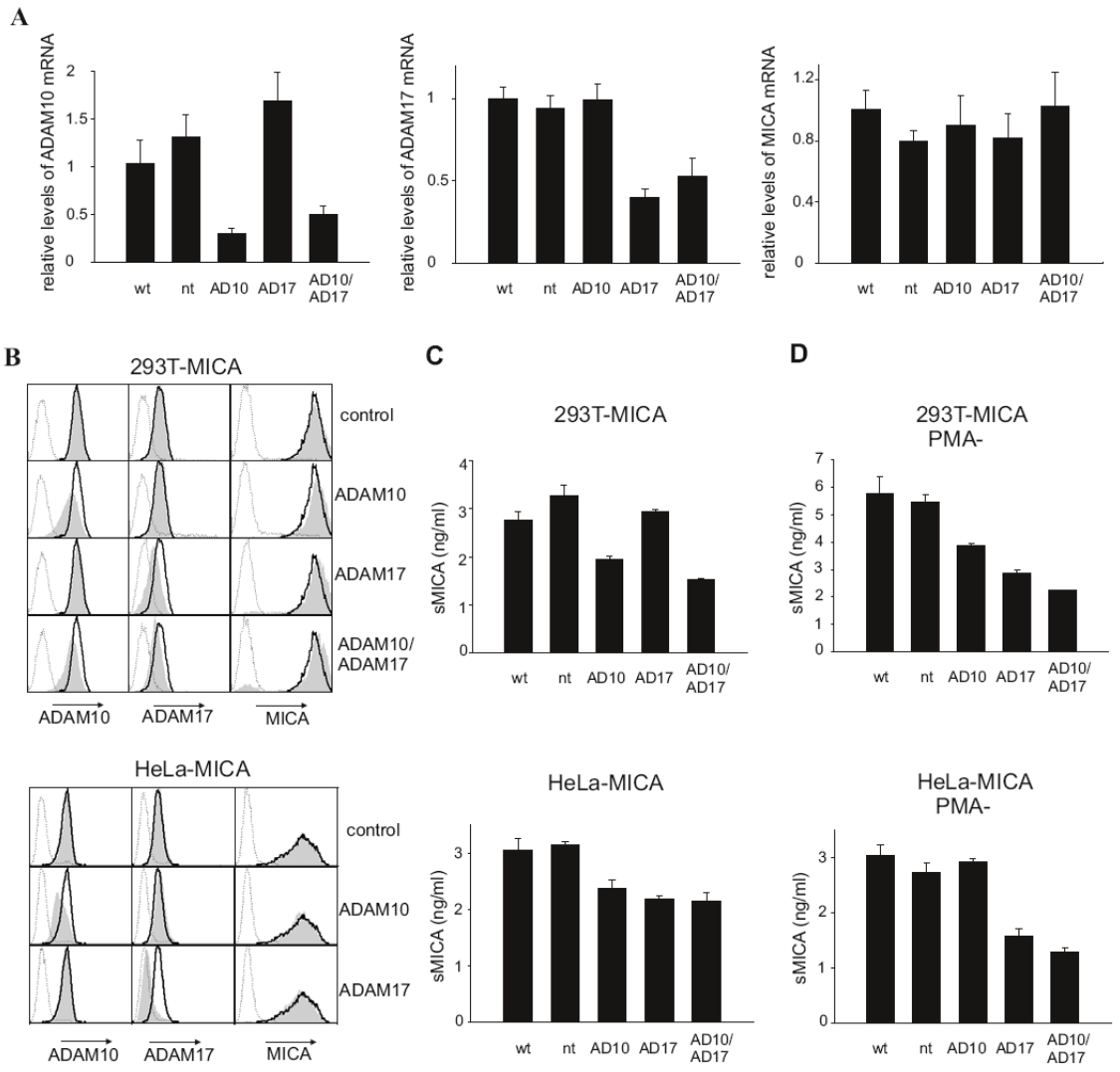

Figure 2.14. ADAM17 mediates PMA-stimulated MICA shedding. (A) Transfection of 293T-MICA with siRNA pools specific for ADAM10 and/or ADAM17 results in markedly reduced levels of ADAM10 (upper) and ADAM17 transcripts (middle), but not MICA transcripts (lower). Relative levels of transcripts of ADAM10, ADAM17 and MICA in 293T-MICA were determined 48 h after transfection with siRNA pools of ADAM10 and/or ADAM17, and a control siRNA pool and compared to levels in untreated 293T-MICA by real-time PCR and normalization with 18S rRNA. **(B)** Transient knockdown of ADAM10 and/or ADAM17 in 293T-MICA and HeLa-MICA by transfection of siRNA results in decreased levels of cell surface ADAM10 and/or ADAM17. Stainings of MICA, ADAM10 and ADAM17 (grey histograms) 72 h after transfection with non-targeting siRNA (control), ADAM10 siRNA, ADAM17 siRNA, or a mixture of ADAM10/ADAM17 siRNA are overlayed with stainings of untreated cells (thick lines) and isotype controls (dotted lines). **(C, D)**. Levels of sMICA were determined in supernatants 72 to 75 h post-transfection **(C)** and after subsequent PMA-treatment (100 ng/ml) at 75 h post-transfection (supernatants of 75 to 77.5 h post-transfection) **(D)**. AD, ADAM; wt, wildtype.

In 293T-MICA cells constitutive MICA shedding was significantly reduced upon knockdown of ADAM10 or a combined knockdown of ADAM10 and ADAM17

knockdown, whereas PMA-stimulated shedding was markedly reduced when either ADAM10 or ADAM17 were silenced **(Figure 2.14)**. Targeting ADAM10 or ADAM17 in HeLa-MICA cells also resulted in a reduced cell surface expression of both ADAM family members **(Figure 2.14B)**. Suppressing ADAM10 resulted in a reduced constitutive MICA shedding, but did not affect PMA-stimulated MICA shedding, whereas silencing ADAM17 reduced PMA-stimulated shedding and constitutive shedding. A combined suppression of ADAM10 and ADAM17 reduced both constitutive as well as PMA-stimulated MICA shedding **(Figure 2.14)**. Altogether, these results demonstrate that both ADAM10 and ADAM17 are critically involved in MICA shedding by tumour cells.

2.3.5 Discussion

MICA shedding is considered a principal mechanism of tumour cells to escape from NKG2D-mediated immunosurveillance in humans. MICA shedding not only results in a reduction of MICA surface density on tumour cells, but also was shown to systemically down-regulate NKG2D on cytotoxic effector cells and to promote expansion of immunosuppressive, intra-tumoural CD4+NKG2D+ T cells [**10,18-20**]. Thus, inhibition of MICA shedding is expected to represent an effective way to improve anti-tumour immunity. Intelligent targeting of the MICA shedding process requires a thorough elucidation of the underlying molecular mechanisms. Previously, we had already shown that shedding of MICA as well as shedding of NKG2DL MICB and ULBP2 can be blocked by broad range metalloprotease inhibitors [**29**]. However, the nature of the proteolytic activities ultimately resulting in release of MICA was not addressed.

Here, we report several lines of evidence that ADAM10 and ADAM17 act as principal sheddases of tumour-cell associated MICA: (i) MICA cleavage occurs at the surface of tumour cells, (ii) MICA is cleaved within the juxtamembranous stalk, (iii) MICA cleavage is dependent on the length, but not on the sequence of the stalk, (iv) MICA shedding is inhibited by broad range metalloprotease inhibitors, but not by MMP-specific TIMP2, (v) MICA shedding is induced by PKC, (vi) constitutive and induced MICA shedding is variably affected by ADAM-specific inhibitors GI254023X and GW280264X, (vii) MICA shedding is suppressed upon silencing of ADAM10 and/or ADAM17.

All these findings are in accord with well-known characteristics of ADAM activities: ADAMs typically release cell surface proteins through cleavage within the stalk region proximal to the membrane and select their substrates not by recognition of consensus sequences, but rather are guided by secondary structures also involving length and accessibility of the stalk [**25,32**]. Activation of PKC has been found to increase ADAM-mediated cleavage events [**31**]. ADAMs are often up-regulated by tumour cells and are critically involved in promoting tumour growth and metastasis. In tumour cells, ADAM10 and ADAM17 provide growth factors by cell surface shedding and thus are essential in promoting cellular growth [**22,26,31**]. Hence, targeting ADAM10 and/or ADAM17 in malignant disease appears promising. In vivo, activity of ADAMs as well as MMPs is inhibited by TIMPs. There exist four TIMPs which block MMPs and ADAMs through binding to the active site, except TIMP2 which does not affect ADAMs [**30**]. It was shown that TIMP3 inhibits tumour growth in vivo [**33**], but many primary tumours lack detectable levels of TIMP3 due to aberrant DNA hypermethylation and this correlates with disease progression [**34**].

Small synthetic hydroxamate-based inhibitors also are able to inhibit ADAMs by blocking the active site [**25**]. Hydroxamate-based inhibitors such as BB94 and Marimastat were previously used in clinical trials [**35,36**], but turned out to be inefficient or caused side effects, presumably by inhibition of MMPs [**37**]. There now exist novel compounds such as GI254023X, GW280264X, INCB7839 and INCB3619 which preferentially inhibit ADAM family members, including ADAM10 and ADAM17 [**27,38**]. The latter are main sheddases of ErbB receptor ligands such as EGF, TGFα, and HB-EGF, important regulators of cell proliferation and survival [**22,31**]. To assess the impact on ErbB-mediated tumour growth, INCB3619 is currently evaluated in preclinical testing [**38**].

Our present study shows that suppression of ectodomain shedding by ADAM10 and ADAM17 also interferes with NKG2DL shedding from tumour cells and thereby is expected to hinder escape from NKG2D-mediated immunosurveillance. Hence, it is of great interest to determine whether treatment with these novel ADAM-specific inhibitors also results in an enhanced tumour immunity against NKG2DL-bearing tumours. Recently, Spies and colleagues demonstrated that the thioreductase ERp5 plays an essential role in MICA shedding [**39**] presumably by chaperoning conformational alterations of surface MICA that may render MICA susceptible for proteolytic cleavage. Presently, it is unclear how activities of ADAMs and ERp5

interrelate in the MICA shedding process, but one may envision that chaperoning functions of ERp5 prepare surface MICA for proteolytic shedding by ADAMs. Further studies, e. g. in animal models are necessary to establish whether targeting ADAMs or ERp5 in vivo can diminish MICA shedding to such an extent where anti-tumour NKG2D reactivity is restored.

Taken together, our study provides strong evidence that ADAM10 and ADAM17 are principal MICA sheddases on tumour cells and therefore represent attractive molecular targets in efforts to improve the efficacy of immunotherapeutic cancer regimen.

2.3.6 Acknowledgements

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2.3.7 References

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2.4 Mutual activation of natural killer cells and monocytes mediated by interaction between the human NK receptor NKp80 and the myeloid-specific receptor AICL

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The author of this thesis performed the AICL Immunoblots **(Figure 2.21f)**.

2.4.1 Abstract

Receptors encoded by the natural killer gene complex (NKC) (e.g. NKG2D) govern reactivity of natural killer (NK) cells. However, the function and ligand(s) of the NKC-encoded human NK receptor NKp80 remain elusive. Here we demonstrated that NKp80 binds to the genetically linked orphan receptor AICL, which like NKp80 is absent in rodents. We defined AICL as a myeloid-specific activating receptor that is up-regulated by Toll-like receptor stimulation. AICL-NKp80 interactions promoted NK cell-mediated cytolysis of malignant myeloid cells. In addition, during cross-talk between NK cells and monocytes, NKp80 stimulated the release of pro-inflammatory cytokines from both cell types. Thus, by specifically bridging NK cells and myeloid cells, NKp80-AICL interactions may contribute to the initiation and maintenance of immune responses at sites of inflammation.

2.4.2 Introduction

Originally, natural killer (NK) cells were primarily considered to be innate immune effector cells capable of spontaneously destroying infected or transformed cells [**1**]. NK cells detect malignant or virus-infected cells through the recognition modes of 'missing-

self' and 'induced-self', which regulate cytokine secretion and cytotoxicity and ultimately lead to the elimination of harmful cells [**2,3**].

Emerging evidence now also attributes to NK cells an important role in the initiation and modulation of adaptive immune responses [**4,5**]. Specifically, by providing an early source of interferon-γ (IFN-γ), NK cells are instrumental in initiating T helper type 1 (TH1) T cell responses [**6**]. However, the molecular and cellular events involving NK cells at the initiation of adaptive immune responses remain unclear. In this regard, the cellular cross-talk between NK cells and myeloid cells is currently of major interest, since it is thought to crucially program subsequent immune responses. Several studies demonstrated a mutual activation of NK cells and dendritic cells (DCs), in which both soluble factors and cell contact-dependent events have been implicated [**5,7-10**]. Other recent reports also define a reciprocal activation between human NK cells and monocytes and macrophages [**11,12**]. Like NK-DC interactions, the stimulatory cross-talk between NK cells and monocytes is partially cell contact-dependent; hence an involvement of hitherto undefined receptors was postulated [**12**].

NK cell activation is balanced by inhibitory signals provided by MHC class I-specific NK receptors (which allow detection of 'missing-self') and activating signals received by several immunoglobulin-like and C-type lectin-like receptors like NKG2D (which allows detection of 'induced self'). In humans, a number of activating NK receptors including NKp30, NKp44, NKp46, NKp80 and NKG2D has been characterized, but apart from NKG2D, the unidentified nature of the corresponding cellular ligands strongly hampers further advances in understanding their role in NK cell-mediated immunoregulation and immunosurveillance [**3,13**].

NKG2D and NKp80 (also called KLRF1) are both C-type lectin-like homodimeric receptors encoded within the human NKC [**14-17**]. NKG2D is expressed by virtually all human NK cells, γδ T cells and $CD8^+$ T cells and, together with the adaptor protein DAP10, assembles into an activating immunoreceptor complex [**3,14**]. Upon recognition of its MHC class I-related, stress-inducible ligands encoded by the MIC and ULBP genes, NKG2D stimulates NK cytotoxicity and cytokine secretion [**14,18**]. Since NKG2D ligands are inducibly expressed in cells subjected to genotoxic stress and are associated with malignant transformation, the hypothesis was put forward that NKG2D may detect and orchestrate the elimination of harmful infected or transformed cells [**3,14,19,20**]. In fact, recent studies illustrate that NKG2D provides protection from spontaneous tumors *in vivo* [**21,22**].

In comparison, much less is known about NKp80 which, unlike NKG2D, has been reported to be expressed exclusively on NK cells [**16**]. In fact, NKp80 was described during the course of a search for novel NK cell-specific surface markers [**26**]. Like NKG2D, NKp80 stimulates NK cell cytotoxicity and induces Ca^{2+} influx in human NK cells upon triggering by appropriate antibodies [**16**]. In contrast to NKG2D, NKp80 lacks charged amino acids in its transmembrane domain (thereby disfavouring association with activating adaptor proteins like CD3ζ, DAP12, DAP10 or FcεRIγ) and consensus activation motifs in its cytoplasmic domain. Also in contrast to NKG2D, NKp80 lacks a homologue in rodents [**17**]; this distinction may have impeded investigation of NKp80 function *in vivo*. Recently, two studies of non-human primates confirmed that NKp80 is an NK cell-specific stimulatory receptor [**23,24**]. However, these reports also were limited by the unknown identity of NKp80 ligand(s).

Driven by the knowledge that characterization of NKG2D ligands profoundly improved our understanding of NK cell activation, we set out to identify ligand(s) of NKp80, the only known NKC-encoded activating receptor exclusively expressed on human NK cells. Here, we identified the NKC-encoded orphan receptor AICL (also called CLEC2B) as a ligand of NKp80. Using newly generated AICL-specific monoclonal antibodies we showed that AICL is a novel myeloid-specific receptor expressed by monocytes, macrophages and granulocytes. Cross-linking of both NKp80 and AICL stimulated secretion of pro-inflammatory cytokines, and in co-cultures of NK cells and monocytes, cytokine release was partially dependent on NKp80 engagement. Hence, our findings suggest that the NKp80-AICL interaction is involved in the activating cross-talk between NK cells and myeloid cells, and thus may influence the initiation and maintenance of immune responses in humans.

2.4.3 Materials and Methods

Cells. Peripheral blood leukocytes of healthy donors were isolated according to the guidelines of and as approved by the local ethic committee and cultured in X-Vivo 15 (Cambrex) with 10% FCS. NK cells were purified by the NK cell isolation kit II and monocytes by the $CD16^+$ Monocyte Isolation Kit (for NK co-culture), by the Monocyte Negative Isolation Kit (for cytokine secretion assays), or by CD14 microbeads (all from Miltenyi). Cell purity was between 90-98%. Granulocytes were isolated as described [**40**]. Monocytes were differentiated to macrophages with 50 ng/ml hM-CSF. Cytokines

were from R&D Systems except hIL-15 and hIL-2 (PromoCell). Monocytes were cultured 24 h with 1 μg/ml LPS from *S. typhimurium,* 50 μg/ml poly(I:C) (Sigma), 1 μM S-[2,3-bis(palmitoyloxy)propyl]-cysteine-$(Lys)_4$ (Pam_2Cys SK4; EMC Microcollections), and 10 ng/ml R-848 (S. Bauer, Munich). NK cells and $CD16^+$ monocytes were co-cultured at 4×10^5 cells/well for 12 h at a 1:1 ratio (total 8×10^5 cells/well) with 100 U IL-2/ml. IL-15 and IL-18 (both at 10 ng/ml), and $F(ab')_2$ of 5D12, 7F12, or NP (4-hydroxy-3-nitrophenylacetyl)-specific IgG_1 (all at 10 μg/ml), respectively, were added where indicated. $CD56^+$ cells were stained for intracellular IFN-γ, and $CD14^{dim}16^+$HLA-DR^{bright} cells for intracellular TNF. To calculate monocyte-dependent increases in frequencies of IFN-γ-producing NK cells, monocyte-dependent increase in presence of NP-specific IgG_1 (Fab')2 was set as 100% ((%IFN-γ-producing NK cells with monocytes) - (%IFN-γ-producing NK cells without monocytes) = 100%). Increase in frequencies of TNF^+ monocytes by co-cultivation with NK cells was calculated accordingly.

NKp80- and AICL-specific monoclonal antibodies. Splenocytes of mice repeatedly immunized with NKp80-ED or AICL-ED, respectively, were fused with P3X63Ag8.653 myeloma cells as described [**41**]. Hybridoma supernatants were screened with mixtures of Jurkat-neo or Jurkat-NKp80 transfectants and mixtures of AICL-ED or LLT1-ED-coated microspheres by immunofluorescence. Immunoglobulins were purified from supernatants with Protein A (Biorad). 5D12, 10E4 and 12D11 antibodies are NKp80-specific, 7F12 and 7G4 antibodies are AICL-specific, and all antibodies are of IgG_1 isotype. Antibodies were labeled using Alexa Fluor 647 carboxylic acid-succinimidyl ester according to the manufacturer's protocol (Molecular Probes). $(Fab')_2$ fragments were generated by pepsin digestion und purified from endotoxin by Triton 114 extraction [**42**]. Endotoxins in mAb and $(Fab')_2$ preparations were tested using a *Limulus* amebocyte lysate assay (QCL-1000, Cambrex) and were below 0.1 EU/μg antibody.

Antibodies. PE-conjugated anti-NKp46 and anti-CD56 were from Immunotech, CD14-FITC and isotype control from Immunotools, CD14-PE-Cy7 and isotype control from BioLegend, anti-NKp46 and anti-TREM-1 from R&D Systems, anti-FLAG M2 from Sigma, anti-penta-His from Qiagen, and goat anti-mouse-Ig-PE conjugate from Jackson Laboratories. The anti-NP IgG_1 mAb was a kind gift from Jörg Kirberg, Max-Planck-

Institute for Immunobiology, Freiburg, Germany. All other antibodies were from BD Biosciences.

Cytotoxicity, degranulation and cytokine analysis. Cytotoxicity was analyzed in a 4 h 51chromium-release assay as described [**41**]. Degranulating NK cells were quantified by analysis of surface CD107a after 6 h incubation with plate-bound antibody in the presence of 10 µg/ml Brefeldin A (Sigma) as described [**43**]. Likewise, frequencies of cytokine-producing NK cells were determined by intracellular staining with PE-anti-IFN-γ after 6 h incubation with plate-bound antibody in the presence of 10 µg/ml Brefeldin A and 100 U IL-2/ml. Ionomycin (Sigma) and PMA (Cell Signaling Technology) were used at concentrations of 1 nM and 10 ng/ml, respectively. TNF levels in supernatants of purified NK cells stimulated for 24 h with plate-bound antibody and 100 U IL-2/ml were determined using ELISA CytoSets from BioSource. TNF in supernatants of purified monocytes was measured after 24 h stimulation with plate-bound, endotoxin-low antibody.

Soluble ectodomains (ED) of C-type lectin-like receptors. Ectodomains of NKp80 (Gln64 through Tyr231), AICL (Lys26 through His149), LLT1 (Ala61 through Val191), and CD161 (Ile66 through Ser225) were expressed in 293T cells transfected with the corresponding cDNA containing an N-terminal BirA-tag and C-terminal c-myc- and six-histidine-tags. EDs were isolated from supernatants of 293T-transfectants by affinity chromatography with anti-c-myc columns and biotinylated using BirA Ligase [**41**] and purified by size exclusion chromatography. Before use, biotinylated EDs were either immobilized on streptavidin-coated microspheres (Bangs Laboratories) or tetramerized using PE- or APC-labeled streptavidin (Molecular Probes).

SPR measurements. Using a BIAcore X apparatus (BIAcore AB) AICL ectodomains were immobilized to CM5 chips by amine coupling. In kinetic analyses (flow rate: 50 µl/min), RU from the control flow cell (imLLT1-ED) were subtracted from RU of the AICL-derivatized surface (black traces) with overlayed gray traces representing fitting of a 1:1 Langmuir model to the association and dissociation phases. In steady-state analyses (15 µl/min) RU from the AICL-derivatized surface were corrected by RU from the non-derivatized control cell. Raw data were analyzed and illustrated using the BIAevaluation software (BIAcore AB).

Immunoblot analysis. Immunoblotting was performed as previously described [**44**]. Treatment with Peptide:*N*-Glycanase F (PNGaseF) (New England Biolabs) was for 1 h at 37°C. Samples were blotted and analyzed with 30 µg 7F12/ml.

Statistical analysis. Statistical analysis was done either using the two-tailed unpaired Student's t test or the nonparametric two-tailed Mann-Whitney Rank Sum test both with $\alpha = 0.050$ and SigmaStat 3.1 software (Systat Software). *P*-values less than 0.04 were considered significant.

Real-time RT-PCR. Total RNA was prepared using TRIZOL (Invitrogen) and reverse transcribed by SuperScript II (Invitrogen). cDNA was amplified with NKp80, AICL and 18S rRNA-specific primer pairs in duplicates (40 cycles, 95°C for 15 s, 60°C for 1 min) using SYBRGreen chemistry on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers were designed to flank an intron, where possible, and specificity was validated using cloned cDNA. Data analysis was by the ΔCT method for relative quantification. Similar amplification efficiencies for NKp80, AICL and 18S were demonstrated by analyzing serial cDNA dilutions with values of the slope of log cDNA amount vs. ΔCT of < 0.1. Oligonucleotide sequences (forward; reverse) were 18S rRNA: 5′-CGGCTACCACATCCAAGGAA-3′; 5′-GCTGGAATTACCGCGGCT-3′; NKp80: 5′- TTCAGTGACGTTGCACTGGT-3′; 5′-CTCCCTGAGAAACCAACAGGA-3′; AICL: 5′-TACCAAATCGTTTGGCATGA-3′; 5′-CTGCAAATCCATTTTCTTTCG-3′. Purity of PCR products was analyzed on 3% agarose gels.

Transfectants. Jurkat cells were transfected by electroporation with an NKp80-hybrid cDNA encoding the cytoplasmic and transmembrane domains of human CD69 (Met1 through Gly70), the NKp80 ectodomain (Gly85 through Tyr231), and a C-terminal FLAG-tag followed by a six-histidine-tag in RSV.5 neo. COS-7 cells were transiently transfected using FuGene6 (Roche) with an AICL hybrid cDNA encompassing the cytoplasmic domain of mouse CD3ζ (Arg52 through Arg164), the transmembrane domain of mouse Ly-49A (Ser40 through Met90), the AICL ectodomain (Lys26 through His149), and a C-terminal FLAG-tag followed by a six-histidine-tag in RSV.5

neo or with a bicistronic expression vector with full-length cDNA of AICL or LLT1 (first cistron) followed by the EGFP cDNA (second cistron).

2.4.4 Results

NKp80 stimulates NK cell degranulation and cytokine release

To analyze NKp80 expression and function, we generated a panel of NKp80-specific monoclonal antibodies by immunizing mice with the NKp80 ectodomain (NKp80-ED). The tagged NKp80-ED construct was expressed in 293T cells and purified from supernatants by affinity chromatography **(Figure 2.15)**.

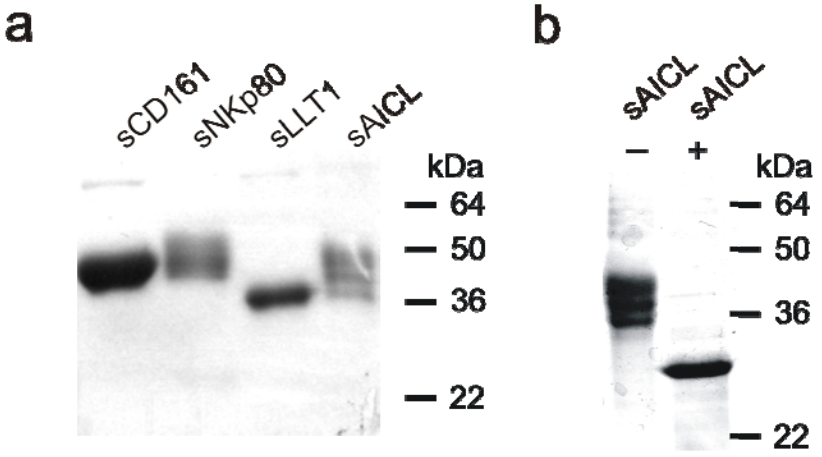

Figure 2.15. Recombinant soluble ectodomains (ED) of various C-type lectin-like receptors. (a) SDS-PAGE of soluble CD161-ED, NKp80-ED, LLT1-ED, and AICL-ED affinity-purified from supernatants of transfected 293T cells. **(b)** SDS-PAGE of soluble AICL-ED untreated (–) or treated (+) with PNGase F.

Specificity of the resulting NKp80-specific antibodies 5D12, 10E4, and 12D11 was verified in binding analyses using microsphere-immobilized NKp80-ED and NKp80-transfected Jurkat cells **(Figure 2.16)**. In accord with previous reports, the NKp80-specific antibody bound to nearly all freshly isolated human NK cells [**16**] **(Figure 2.17a)**. We also noted that the $CD56^{bright}$ NK subset, which is a primary source of monokine-stimulated NK cell cytokine production [**25**], also expressed high amounts of NKp80. NKp80 expression has also been reported to be expressed on $CD3^{+}CD56^{+}$ cells from some donors [**16**]. Accordingly, we found NKp80 on varying fractions of $CD56^{+}CD3^{+}$ cells (range 29-61%, median 43%) **(Figure 2.17b)**.

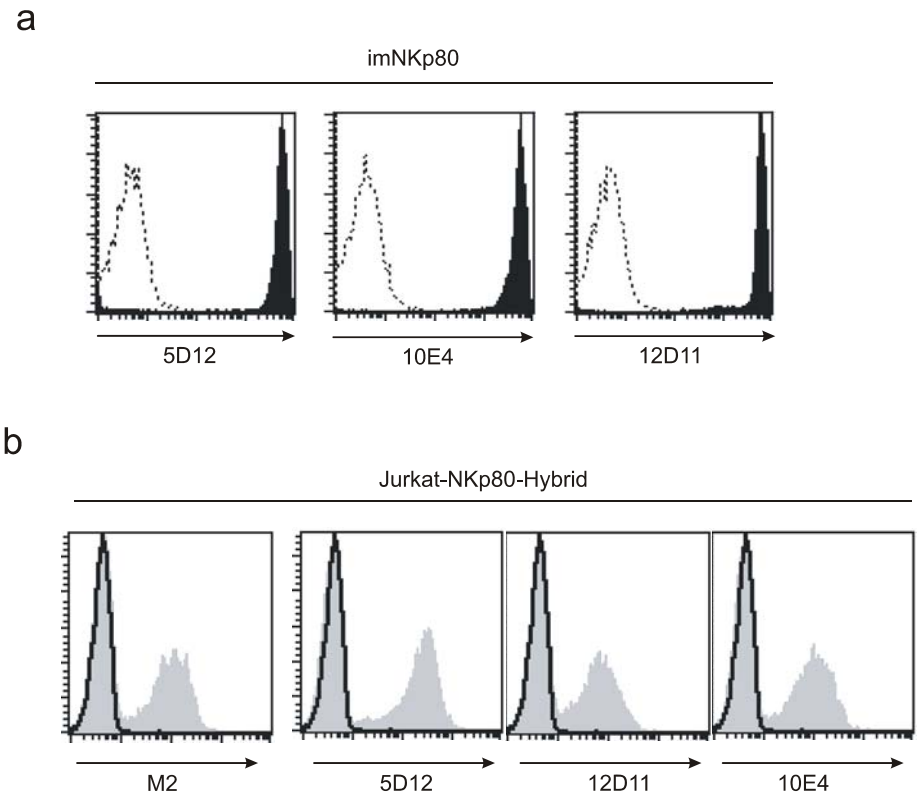

Figure 2.16. Specificity of NKp80-specific antibodies 5D12, 10E4, and 12D11. (a) 5D12, 10E4, and 12D11 binding to microsphere-immobilized NKp80 (imNKp80) (filled histograms) and imLLT1 (open histograms). **(b)** 5D12, 10E4, 12D11, and anti-FLAG binding to a mixture of Jurkat cells transfected with the FLAG-tagged NKp80-CD69 hybrid cDNA and NKp80-hybrid-negative Jurkat transfectants (grey histograms). Black line represents the isotype control staining.

However, we also detected NKp80 on a substantial proportion of γδ T cells. This expression varied widely among individual donors (range 16%-70%, median 26%), and likely accounted for the few NKp80$^+$ CD56$^-$CD3$^+$ T cells **(Figure 2.17b)**. In contrast, B cells, monocytes, and other T cells subsets including CD4$^+$ and CD8$^+$ αβ T cells (CD3$^+$CD56$^-$) were devoid of surface NKp80, as were all tested cell lines (data not shown). These findings were supported by real-time RT-PCR analyses, which revealed a high abundance of transcripts encoding NKp80 in NK cells **(Figure 2.18)**.

The impact of NKp80 triggering on cytokine release by NK cells has not yet been addressed. To investigate the consequences of NKp80 triggering on NK cell effector functions independently of other NK receptors, we incubated freshly purified NK cells with plate-bound anti-NKp80 NKp80 cross-linking triggered secretion of TNF **(Figure 2.17c)**. Simultaneous stimulation with anti-NKp80 and anti-NKp46 further amplified TNF secretion. Similar results were obtained when assessing NK cell IFN-γ production **(Figure 2.17d)**.

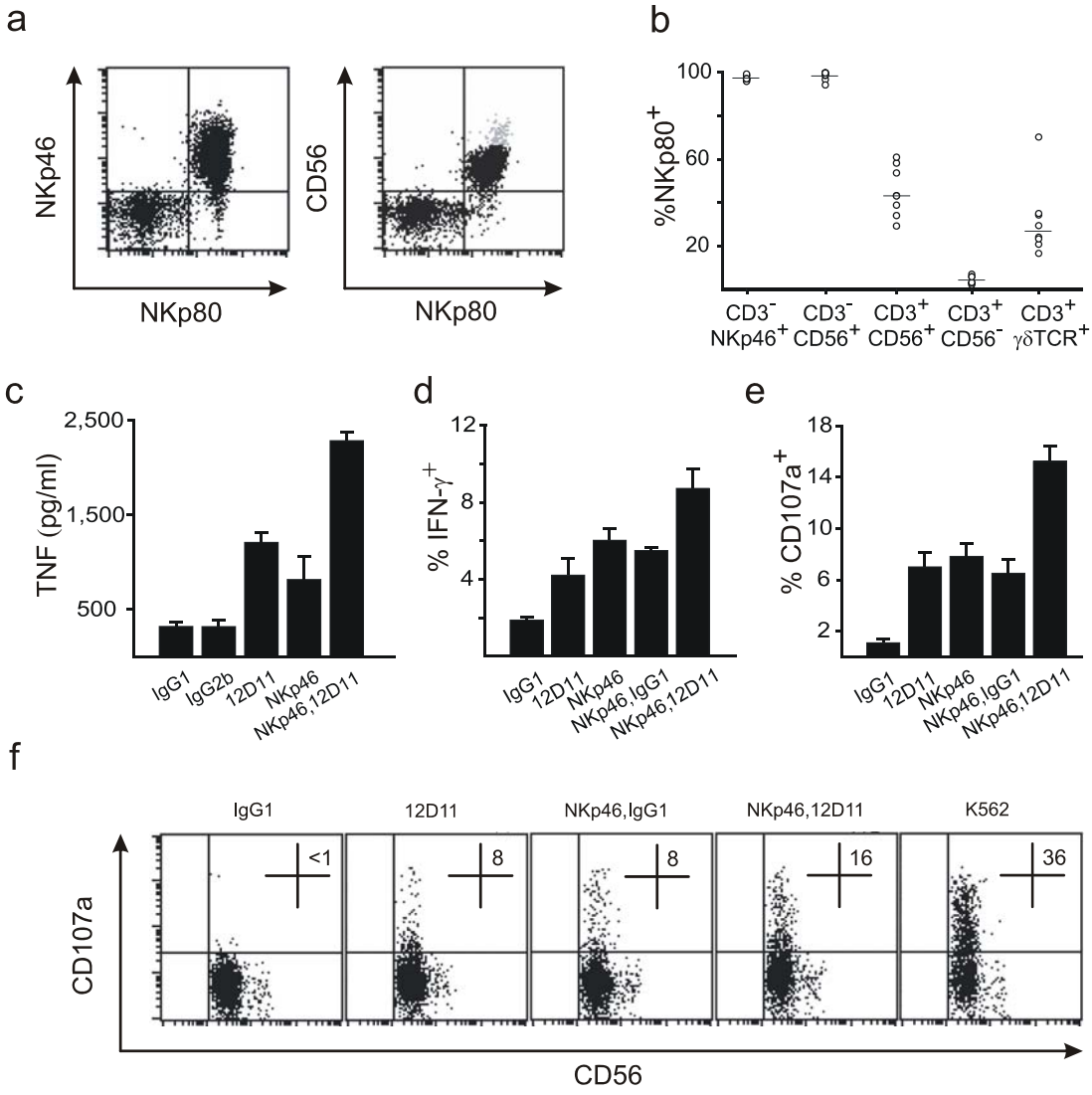

Figure 2.17. NKp80 stimulates granule exocytosis and cytokine secretion. (a) Expression of the indicated molecules on the surface of human resting NK cells was determined by flow cytometry. Plots depict freshly isolated PBMC where $CD3^+$ cells were excluded by electronic gating. **(b)** Frequencies of $NKp80^+$ cells among indicated subpopulations from eight healthy donors. Medians are indicated by horizontal bars, and each dot depicts one individual donor. **(c-f)** Freshly purified NK cells were incubated with the indicated plate-bound antibodies. **(c)** Concentrations of TNF in culture supernatants were determined by ELISA. Results depict means of triplicate samples, and error bars represent s.d. Results are representative of 3 independent experiments. **(d, e)** Frequencies of $IFN\gamma^+$ cells **(d)** and $CD107a^+$ cells **(e)** among $CD56^+$ NK cells were determined by flow cytometry. Results are shown as means of triplicates with s.d. **(f)** Representative analysis of $CD107a^+$ NK cells after stimulation with indicated immobilized antibodies or K562 cells. Percentages of $CD107a^+$ cells of all $CD56^+$ NK cells (upper right quadrant) are depicted. Results in **(d-f)** are representative of 6 independent experiments.

In accordance with published data on NKp80-mediated stimulation of NK cell cytotoxicity [**16**], we observed that immobilized anti-NKp80 also induced enhanced cell

surface exposure of CD107a (also called lysosomal-associated membrane protein (LAMP-1)) which is indicative of NK cell degranulation, in a manner similar to anti-NKp46, again with a pronounced cooperative effect of simultaneous ligation of both receptors **(Figure 2.17e, f)**.

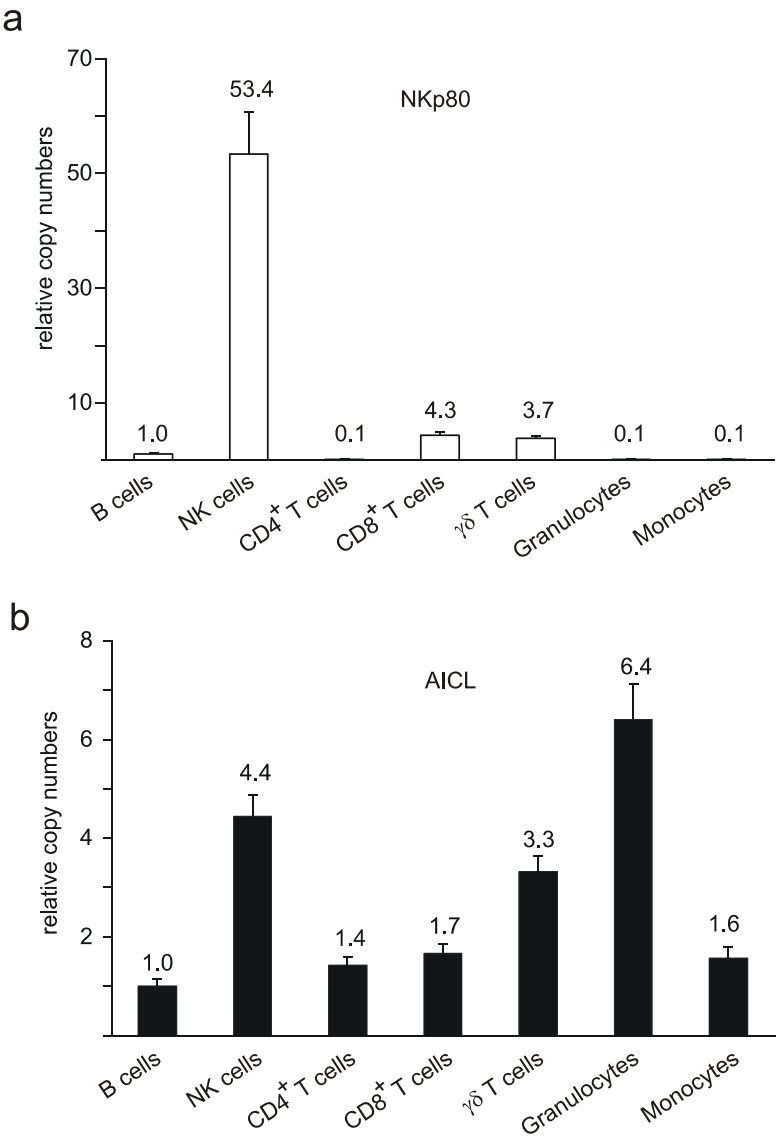

Figure 2.18. Abundance of AICL and NKp80 transcripts in leukocyte subpopulations. (a, b) $CD3^+CD8^+$ ($CD8^+$ T cells), $CD3^+CD4^+$ ($CD4^+$ T cells), $CD3^+\gamma\delta$ TCR^+ (γδ T cells), $CD19^+$ (B cells), $CD66b^+$ (granulocytes), $CD14^+$ (monocytes) and $CD3–CD56^+$ (NK cells) cells were sorted from the peripheral blood of a healthy donor. ΔC_T values for NKp80 **(a)** and AICL **(b)** transcripts were calculated by normalization with 18S RNA and relative copy numbers were determined by setting the ΔC_T value of B cells as 1.

NKp80 engages AICL

Further elucidation of the immunological relevance of NKp80-mediated NK cell activation necessitated the identification of NKp80 ligands (NKp80-L). We attempted to identify NKp80-L bearing cells by using BWZ.36 cells expressing NKp80-CD3ζ reporter constructs, because ligands of the NKC-encoded mouse Nkrp1 receptors were previously identified using BWZ.36 cells expressing Nkrp1-CD3ζ reporter constructs [**26,27**]. These reports revealed that mouse Nkrp1 receptors and their ligands, called C-type lectin-related (Clr) molecules, are all encoded in close genetic linkage within the NKC [**17,26,27**].

Because this strategy failed to identify NKp80-L expressing cells, we considered the possibility that, like Nkrp1-Clr receptor-ligand pairs, the orphan genes encoding Lectin-Like Transcript 1 (LLT1) and Activation-Induced C-type Lectin (AICL), which are located in close proximity to the gene encoding NKp80 in the human NKC, might be ligands of NKp80 **(Figure 2.19a)**. In fact, while this work was in progress, LLT1 was reported as a ligand of the single human representative of the Nkrp1 receptor family, NKR-P1A (also called CD161) [**28,29**]; this receptor-ligand pair is also genetically linked within the NKC. In contrast to CD161, no known mouse homologues of NKp80 or for AICL have been identified [**17**]. Hence, to directly assay a possible interaction between NKp80 and AICL or LLT1, we produced soluble ectodomains of AICL (AICL-ED) and LLT1 (LLT1-ED) using stably transfected 293T cells **(Figure 2.15)**. AICL-ED or LLT1-ED, respectively, were immobilized on streptavidin-coated microspheres and directly tested for binding to fluorochrome-labeled NKp80-ED or CD161-ED tetramers, respectively, via flow cytometry. As expected, CD161-ED-tetramers bound immobilized LLT1, although staining was fairly weak, indicating a low affinity interaction in agreement with recent reports [**28,29**] **(Figure 2.19b)**. In contrast, NKp80-ED-tetramers did not bind to LLT1, but exhibited strong binding to immobilized AICL **(Figure 2.19b)**. Similar results were obtained in a ‘reverse’ setting in which immobilized NKp80-ED specifically interacted with AICL-ED-tetramers **(Figure 2.19c)**. These data suggest that AICL, but not LLT1, is a ligand for NKp80.

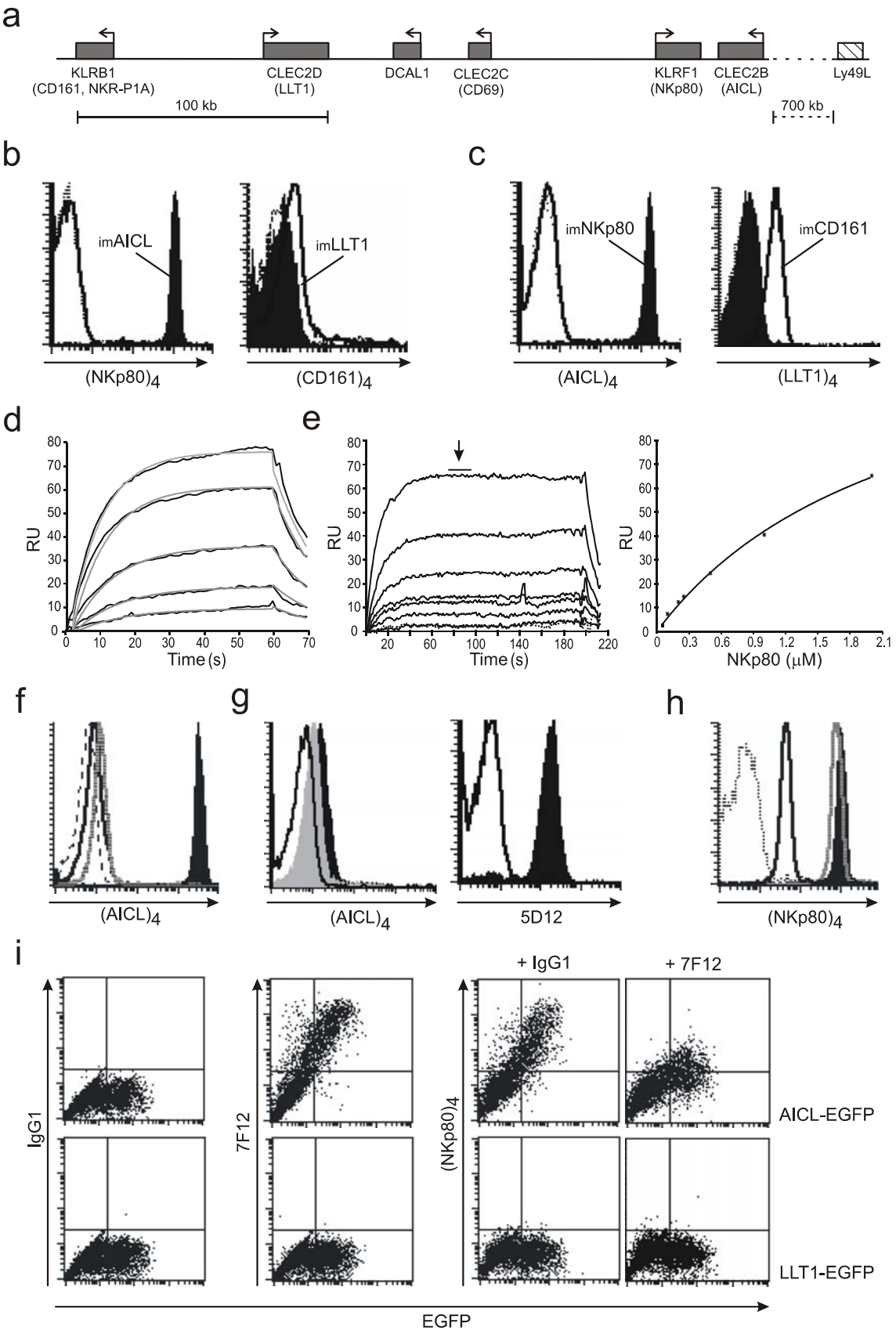

Figure 2.19. NKp80 engages AICL. (a) Scheme of a subregion of the human NKC with *KLRF1* and *CLEC2B* encoding NKp80 and AICL, respectively. Ly49L marks the centromeric end of the NKC. Boxes and arrows represent genes and transcriptional orientation, respectively. **(b)** Staining of immobilized sAICL-ED (imAICL) (black fill), imLLT1 (black line) or imNKp80 (dashed line) with indicated tetramers. **(c)** Staining of

imNKp80 (black fill), imCD161 (black line), imAICL (dashed line, left panel) or imLLT1 (dashed line, right panel) with indicated tetramers. **(d)** Kinetics of binding of sNKp80-ED (0.12, 0.25, 0.5, 1.0, 2.0 μM) to immobilized AICL-ED as described in Methods. RU, resonance units. **(e)** Affinity of NKp80 to AICL was determined by injecting sNKp80-ED (0.05, 0.1, 0.2, 0.25, 0.5, 1.0, 2.0 μM) over immobilized AICL (left panel). Dotted trace indicates control injection (2μM sCD161-ED) and arrow averaged interval for a best-fit curve (right panel) **(f)** Binding of sAICL-ED tetramer to NKp80 after pre-incubation with 5D12 (gray line) and 10E4 (dashed line), IgG_1 isotype control (black fill), or to imAICL (black line). **(g)** Left, binding of sAICL-ED tetramers to NK cells after pre-incubation with 10E4 (gray fill) and IgG_1 control (black fill) or of sCD161-ED tetramers (black line) to NK cells. Right, surface NKp80 detected by 5D12 (black fill) and isotype control (black line). **(h)** Binding of sNKp80-ED tetramers to imAICL after incubation with 7F12 (black line) and 7G4 (gray line) or IgG_1 isotype control (black fill). Staining of imNKp80 served as negative control (dotted line). **(i)** Binding of indicated reagents to COS-7 cells transfected with indicated bicistronic EGFP constructs after pre-incubation with indicated antibodies. Data **(b-i)** are representative of at least two independent experiments.

Using surface plasmon resonance (SPR) technology, we determined the affinity of the NKp80-AICL interaction. Soluble NKp80 ectodomains bound to immobilized AICL-ED with an intermediate association rate (k_{on} = 1.6 × 10^4 $M^{-1}s^{-1}$) and dissociated relatively rapidly (k_{off} = 6.7 × 10^{-2} s^{-1}) **(Figure 2.19d)**. The affinity for the NKp80-AICL interaction calculated from on- and off-rates ($K_{D,calc}$ = 4.1 μM at 25°C) is comparable to the affinity determined during steady-state analyses (K_D ~ 2.3 μM at 25°C) **(Figure 2.19e)**. Pre-incubation of NKp80-ED-coated microspheres with various NKp80-specific antibodies blocked binding of AICL-ED-tetramers **(Figure 2.19f)**. Importantly, AICL-ED-tetramers also stained freshly isolated NK cells and binding was blocked by pre-treatment of NK cells with NKp80-specific antibodies, demonstrating that AICL is a natural ligand of NKp80 **(Figure 2.19g)**.

AICL is a myeloid-specific surface receptor

A single study reported differential expression of AICL mRNA expression in T and B lymphocytes, monocytes and granulocytes [**30**]. By real-time PCR, we confirmed that AICL transcripts were most abundantly expressed in granulocytes, and found these more prominently in NK cells and γδ T cells than in αβ T cells or B cells **(Figure 2.18)**. However, due to a prior lack of AICL-specific antibodies, AICL protein expression was not examined in previous studies. Thus, to explore AICL expression, we generated AICL-specific antibodies by immunizing mice with AICL-ED. Two antibodies, 7F12 and 7G4, bound immobilized AICL-ED, but not LLT1-ED, NKp80-ED or CD161-ED; these antibodies also stained COS-7 cells transiently transfected with AICL-Ly49A-

CD3ζ hybrid constructs, in which transmembrane and cytoplasmic sequences of AICL were replaced by those of mouse Ly49A (transmembrane) and mouse CD3ζ (cytoplasmic) sequences **(Figure 2.20,** and data not shown).

Pre-incubation of microsphere-immobilized AICL with 7F12, but not with 7G4, reduced binding of NKp80-ED tetramers indicating that 7F12 partially hinders the NKp80-AICL interaction **(Figure 2.19h)**. Importantly, NKp80-ED tetramers also bound AICL expressed on the surface of transfected COS-7 cells, and addition of 7F12 interfered with binding **(Figure 2.19i** and **Figure 2.20)**.
Next, we analyzed AICL surface expression on various cell lines, and detected AICL on the surface of myeloid cell lines U937, THP-1 and MEG-01 **(Figure 2.21a** and **Table 2.3)**. U937 cells, which expressed the highest amounts of AICL, also bound high amounts of NKp80-ED tetramers, and pre-incubation with 7F12 markedly reduced NKp80-ED binding **(Figure 2.21b)**.
In contrast to myeloid cell lines, AICL was not detectable on non-myeloid hematopoietic or on non-hematopoietic cell lines **(Figure 2.21a** and **Table 2.3)** suggesting that AICL is preferentially expressed on the surface of myeloid cells.

Thus, we analyzed AICL expression on peripheral blood leukocytes and observed specific binding of 7F12 and 7G4 to monocytes, macrophages and granulocytes, but not to T cells, B cells, or NK cells **(Figure 2.21c, d** and **Figure 2.20)**. Among monocytes, the $CD14^{dim}CD16^{+}$ subset, which is a major source of TNF [**31**] exhibited substantially higher AICL surface expression than the $CD14^{bright}CD16^{-}$ subset **(Figure 2.21d)**.

We also assessed AICL expression on DCs, because the cellular cross-talk between NK cells and DCs has attained much interest [**5**]. Interestingly, AICL expression decreased when monocytes were differentiated *in vitro* to immature DCs **(Figure 2.21e)** indicating that NKp80-AICL interactions may not be involved in the interaction of NK cells with monocyte-derived DCs. A previous report suggested that NKp80-L may be expressed on activated T cells, because NK cell-mediated cytotoxicity against PHA-activated T cells was partially reduced by addition of NKp80-specific antibodies [**42**]. However, we were unable to detect AICL on the surface of activated T cells **(Table 2.4)**. Myeloid-specific AICL expression was surprising given that a previous report [**30**] and our analyses detected AICL transcripts also in lymphocytes.

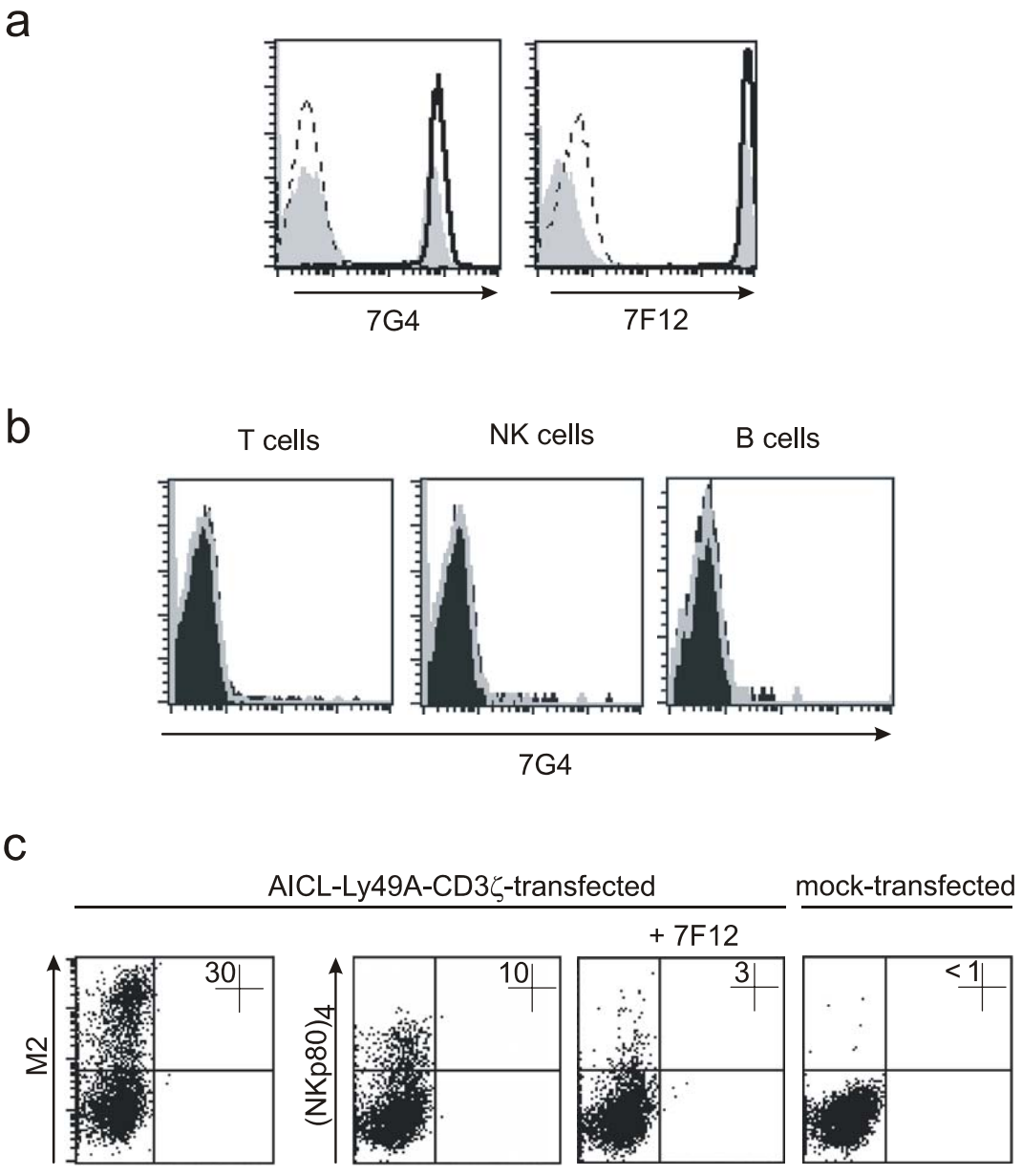

Figure 2.20. (a) 7F12 and 7G4 binding to microsphere-immobilized AICL (imAICL) (solid line), to imNKp80 (dashed line), and to a 2:1 mixture of imNKp80-microspheres and imAICL-microspheres (gray histograms). **(b)** Staining of 7G4 of freshly isolated T cells ($CD3^+$), NK cells ($CD56^+$) and B cells ($CD19^+$) (filled histogram). Open histograms represent isotype control stainings. **(c)** NKp80-ED tetramer binding to COS-7 cells transiently transfected with an AICL-Ly49A-CD3ζ hybrid after pre-incubation with or without 7F12. NKp80-ED tetramer binding to mock-transfected COS-7 cells is indicated. AICL-hybrid expression was monitored by staining with the FLAG-specific antibody M2. Percentages of stained cells (upper left quadrant) are given.

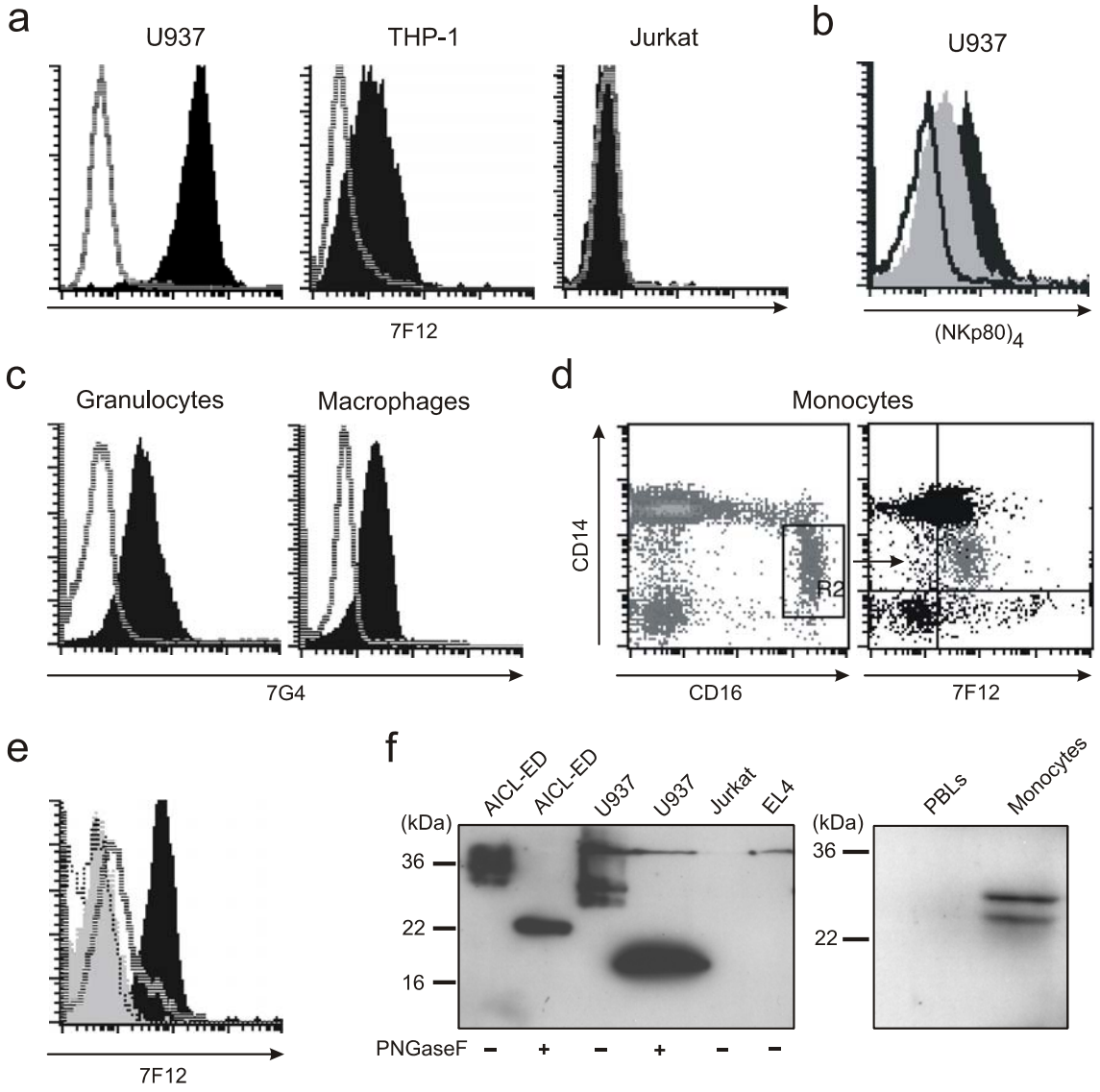

Figure 2.21. AICL is a myeloid-specifc receptor. (a) Staining with AICL-specific antibody 7F12 (black fill) or IgG_1 isotype control (gray line) on myeloid cell lines U937 and THP-1 and the T cell line Jurkat, as determined by flow cytometry. **(b)** Binding of NKp80-ED tetramers to U937 cells with and without pre-incubation with 7F12 (gray fill) or IgG_1 isotype control (black fill). Negative control, staining with PE-conjugated streptavidin (black line). **(c)** Staining of freshly isolated granulocytes and in vitro matured macrophages with AICL-specific antibody 7G4 (black fill) and IgG_1 isotype control (gray line). **(d)** Expression of indicated molecules on the surface of freshly isolated monocytes. Gray cells in right panel depict cells within R2 in left panel. **(e)** AICL expression on purified monocytes at day 0 (black fill) or after 6 days culture with GM-CSF and IL-4 (gray line). IgG_1 isotype control stainings at day 0 (dashed line) and day 6 (gray fill) are indicated. **(f)** AICL in lysates of indicated cell lines (left), freshly isolated monocytes and lymphocytes (PBLs) (right) was detected by immunoblotting with 7F12. Lysates were deglycosylated with PNGase F where indicated. Recombinant AICL-ED is included as positive control. Data **(a-f)** are representative of at least two independent experiments.

Therefore we analyzed AICL protein in whole cell lysates using 7F12 and detected AICL in lysates of U937 cells and monocytes, but not in lysates of non-myeloid cell lines or lymphocytes **(Figure 2.21f)**. Together these data define AICL as a myeloid-specific surface receptor capable of binding NKp80 on NK cells.

Table 2.3. **AICL surface expression on human primary cells and tumour cell lines.** Primary cells and tumour cell lines were analyzed by flow cytometry for reactivity with AICL-specific antibodies 7F12 and 7G4. Mean fluorescence intensity (MFI): to 10 (-); 10 to 15 (+/-); 15 to 50 (+); 50 to 200 (++), above 200 (+++). nd (not done).

Cells	Histotype	7F12	7G4
Resting NK cells		-	-
Activated NK cells		-	-
Resting T cells		-	-
PHA blasts		-	-
Resting B cells		-	-
Monocytes		+	+
LPS-activated monocytes		++	++
Granulocytes		+	+
Macrophages		+	+
immature monocyte-derived DC		-/+	nd
mature monocyte-derived DC		-	nd
YT	NK cell line	-	-/+
NKL	NK cell line	-	-
CEM	T leukemia	-	-
MOLT4	T leukemia	-	-
Jurkat	T leukemia	-	-
HBP	EBV-transformed B cell line	-	-
LCL 721.221	EBV-transformed B cell line	-	-
WT51	LCL	-	-
T1	B-LCL 721.174xCEMR.3	-	-
C1R	EBV-transformed B cell line	-	-
RPMI 8866	EBV-transformed B cell line	-	-
K562	erythroleukemia	-	-
THP-1	acute monocytic leukemia	+	+
MEG-01	chronic myelogenous leukemia	++	++
U937	human histiocytic lymphoma	+++	+++
HL60	acute promyelocytic leukemia	-	-
NB4	acute promyelocytic leukemia	-	-
293T	embryonic fibroblasts	-	-
AML-01	acute myeloid leukemia	-	-

BV173	chronic myeloid leukemia	-	-
KYO-1	chronic myeloid leukemia	-/+	-/+
CaCo-2	colon carcinoma	-	-
MelJuso	melanoma	-	-
Ma-Mel-8a	melanoma	-	-
HCT116	colon carcinoma	-	-
SW756	cervix carcinoma	-	-
MG63	osteosarcoma	-	-
WEHI-3B	mouse myelomonocytic leukemia	-	-

AICL triggers monocyte cytokine release

Ligands of Toll-like receptors (TLRs) modulate the cell surface expression of various immunoreceptors, including TREM-1, CD80 and CD83 [**32**]. Hence, we determined whether TLR stimulation modulated AICL surface expression. AICL was markedly up-regulated within 24 h of exposure of monocytes to the TLR ligands LPS, poly (I:C), R848, or Pam_2Cys SK4 **(Figure 2.22a** and data not shown). In accord with the lack of TLR9 expression by human monocytes stimulation with the TLR9 ligand CpG DNA did not affect AICL surface expression.

Next, we determined whether AICL ligation could stimulate monocytes. Like stimulation with LPS or with TREM-1-specific antibodies, AICL cross-linking enhanced monocyte TNF production **(Figure 2.22b)**. In addition, LPS exerted a strong additive effect on AICL-stimulated TNF release **(Figure 2.22c)**.

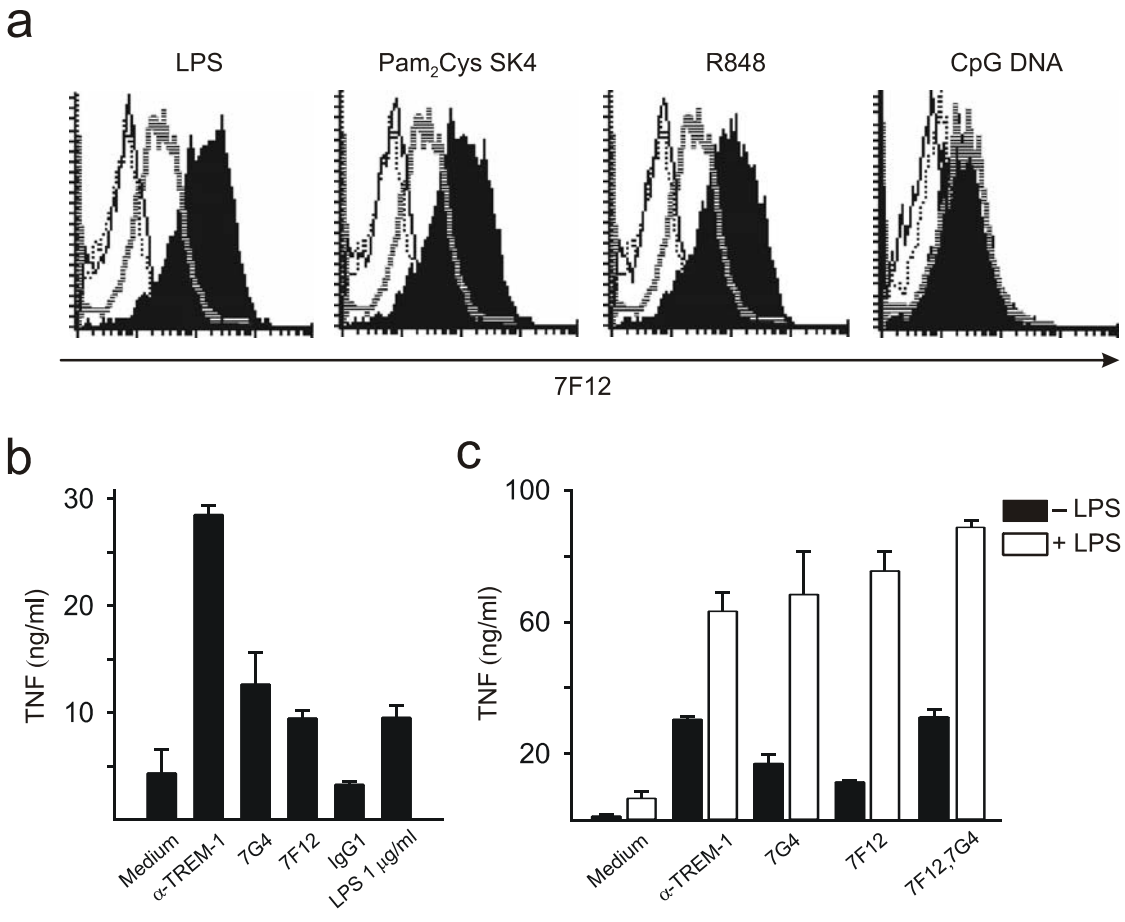

Figure 2.22. AICL is up-regulated by TLR stimulation and stimulates TNF release. **(a)** Freshly purified monocytes stimulated for 24 h with indicated TLR ligands were stained with AICL-specific antibody 7F12 (black fill) or IgG_1 isotype control (dotted line). Stainings of mock-treated monocytes with 7F12 (gray line) or IgG_1 isotype control (black line) are shown. **(b)** TNF in supernatants of freshly isolated monocytes cultivated for 24 h with indicated plate-bound antibodies was measured by ELISA. **(c)** TNF in supernatants of freshly isolated monocytes stimulated for 24 h with indicated plate-bound antibodies in the presence (open bars) or absence of LPS (black bars) was measured by ELISA. In **b** and **c** means of triplicates are shown, error bars represent s.d. All results are representative of at least 3 independent experiments.

NKp80 promotes lysis of AICL$^+$ target cells

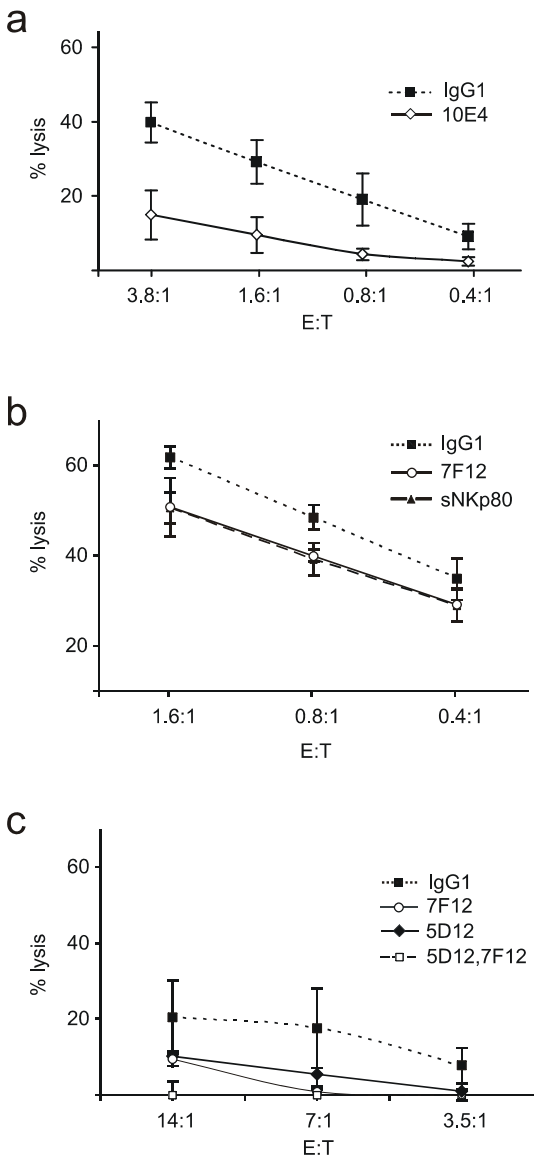

Figure 2.23. NKp80-AICL interaction promotes NK cell-mediated cytolysis of myeloid cells. (a-c) Cytotoxic activity of freshly purified NK cells was measured during a 4 h 51chromium-release assay **(a**, **b)** Lysis of U937 cells in presence of indicated antibodies. Results are representative of four **(a)** and two **(b)** independent experiments. **(c)** Lysis of LPS-activated, CD14$^+$ monocytes in the presence of indicated antibodies. Results are representative of two independent experiments with cells from different donors. F(ab')$_2$ fragments were used in all experiments. NK cells in **(a-c)** were from different donors with data depicted as means of quadruplicate **(a**, **b)** or triplicate **(c)** samples. Errors bars represent s.d.

Previous studies demonstrated that NKp80 stimulates NK cytotoxicity in redirected lysis assays when cross-linked by NKp80-specific antibodies [**16,23,24**]. However, due to the unknown nature of NKp80-L, the importance of NKp80-dependent cytotoxicity in a biologically relevant setting could not be assessed. Here, we addressed the impact

of NKp80 on NK cell cytotoxicity towards myeloid cells expressing AICL. U937 cells express high amounts of AICL, but also of ligands for the activating NK receptor DNAM-1 [**33**].

Accordingly, freshly isolated NK cells strongly lysed U937. U937 lysis was partially blocked by anti-NKp80 10E4, suggesting that NKp80 markedly contributes to NK cell-mediated cytolysis of U937 cells **(Figure 2.23a)**. Furthermore, addition of either 7F12 or soluble NKp80 also reduced NK cytotoxicity against U937 cells **(Figure 2.23b)**. In contrast to U937 cells, non-malignant myeloid cells like monocytes express low amounts of AICL and DNAM-1 ligands and are largely resistant to NK cell-mediated cytolysis (data not shown). However, after 24 h of LPS treatment, in two out of four donors, we observed moderate NK cell-mediated cytolysis of autologous monocytes, which was inhibited by treatment with NKp80- and AICL-specific antibodies **(Figure 2.23c** and data not shown). These data indicate that TLR-mediated activation may render monocytes susceptible to NKp80-dependent NK cell-mediated cytolysis.

NKp80-dependent NK-monocyte cross-talk

A recent report [**12**] described a bi-directional activation pathway between NK cells and monocytes that results in secretion of IFN-γ and TNF by NK cells and monocytes, respectively. It was suggested that this mutual activation may occur at sites of inflammation, particularly during chronic inflammatory autoimmune diseases when activated $CD56^{bright}$ NK cells and monocytes are prominent [**12**]. Co-culture of NK cells and monocytes in the presence of monokines results in increased secretion of pro-inflammatory cytokines by NK cells and monocytes, and this increase is partially dependent on cell contact [**12**]. However, the receptors involved in this cell contact-dependent NK cell-monocyte cross-talk remain unidentified.

Here we here adopted this same experimental system to confirm that co-culture of freshly isolated autologous NK cells and monocytes results in increased frequencies of IFN-γ-secreting NK cells and TNF-secreting monocytes, respectively, as compared to cultures of either NK cells or monocytes alone **(Figure 2.24)**. In accordance with previous studies, $CD56^{bright}$ NK cells were more prone to produce IFN-γ than $CD56^{dim}$ NK cells [**25**] **(Figure 2.24b** and **Table 2.4)**. Importantly, addition of IL-15 and IL-18 monokines was essential to induce cytokine secretion.

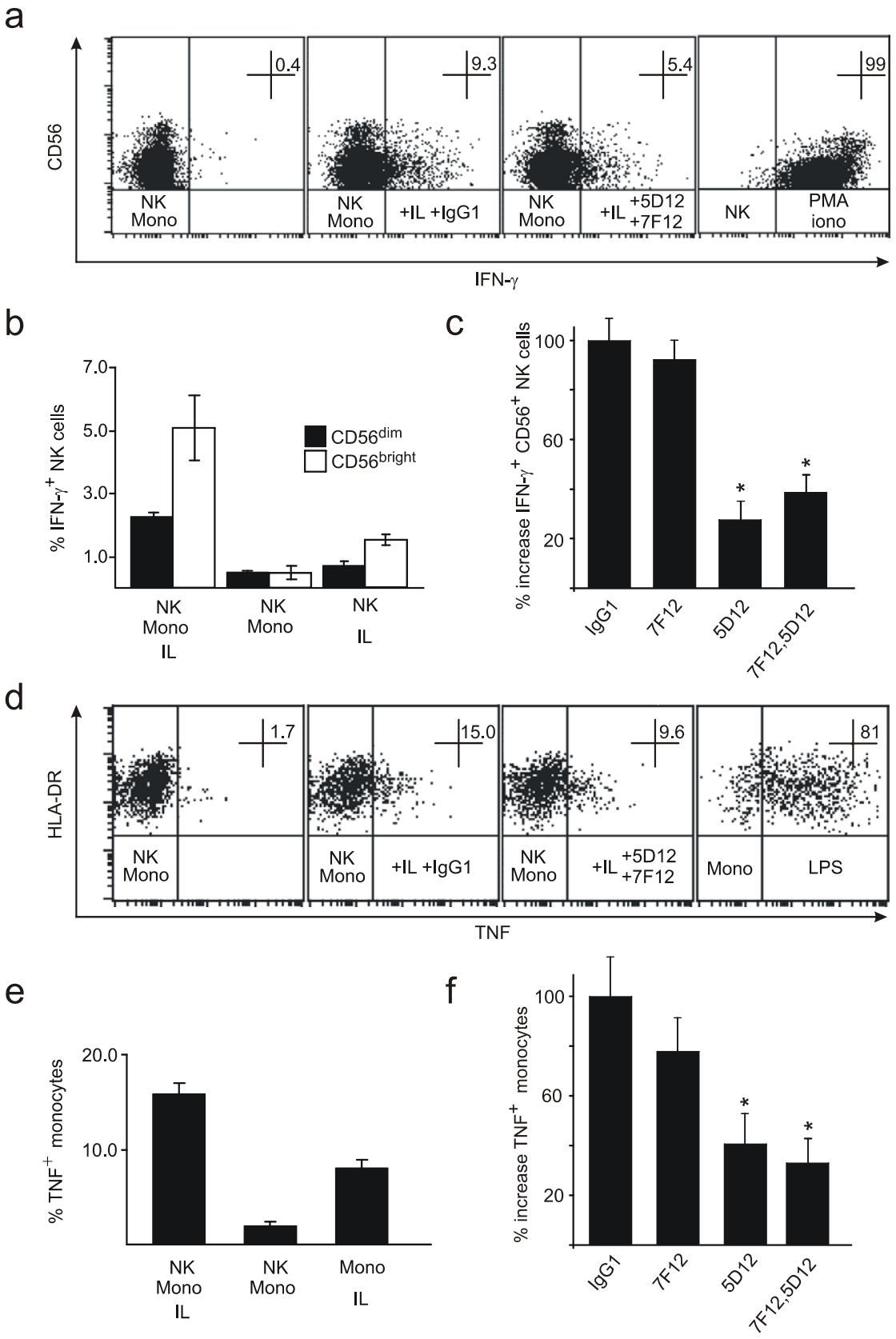

Figure 2.24. NKp80-dependent stimulation of cytokine release. (a-c) Frequency of IFN-γ-producing NK cells after 12 h culture with autologous CD14dimCD16$^+$ monocytes. **(a)** Representative analysis of NK cells cultured with monocytes (Mono) or

monokines (IL) in the presence of indicated antibodies. Stimulation with PMA and ionomycin served as a positive control (right panel). Percentages of IFN-γ^+ cells of all $CD56^+$NK cells (upper right quadrant) are indicated. **(b)** Frequency of IFN-γ^+CD56dim NK cells (black bars) or IFNγ^+CD56bright NK cells (open bars) after culture with monocytes, monokines or both. **(c)** Change in frequency of IFN-γ^+ NK cells after co-culture with monocytes in the presence of monokines and indicated antibodies. **(d-f)** Frequency of $TNF^+CD14dimCD16^+$ monocytes after 12 h culture with autologous NK cells. **(d)** Representative analysis of monocytes cultured with NK cells in the presence of monokines and indicated antibodies. Stimulation with LPS served as a positive control (right panel). Percentages of TNF^+ cells of all HLA-DR^+ monocytes (upper right quadrant) are indicated. **(e)** Frequency of TNF^+ monocytes after culture with NK cells, monokines or both. **(f)** Change in frequency of TNF^+ monocytes after culture with NK cells in the presence of monokines and indicated antibodies. In **c** and **f** the frequency of cytokine-producing cells after culture with isotype control IgG_1 is set as 100% (for details see Methods). All data are means of triplicate (**a**, donor 6) or quadruplicate (**b-f**, donor 3) samples, and errors bars represent s.d. *P*-values were calculated using the two-tailed Student's t test and * indicates a significant difference ($P < 0.001$) to the IgG_1 isotype control.

To investigate whether NKp80-AICL interactions may contribute to this cell contact-dependent NK cell-monocyte cross-talk, we added $F(ab')_2$ fragments of the NKp80-specific antibody 5D12 and/or the AICL-specific antibody 7F12 to NK cell-monocyte co-cultures. Blockade of NKp80-AICL interactions strongly reduced the monocyte-dependent increase in NK cell IFN-γ secretion, demonstrating that NKp80-AICL interactions are crucially involved in the activating NK-monocyte crosstalk **(Figure 2.24a, c)**. Although the frequencies of IFN-γ-secreting $CD56^{bright}$ NK cells varied widely between various donors (range 3.8% to 40.2%), NKp80 blockade always resulted in a strong reduction of responsive cells **(Table 2.4)**. Similarly, frequencies of IFN-γ-secreting $CD56^{dim}$ NK cells (range 1.7% to 25.2 %) were markedly reduced in four out of five donors analyzed. In contrast, 7F12 did not significantly affect IFN-γ-secretion by NK cells, presumably due to its inefficient blocking capability. Conversely, enhanced TNF secretion by monocytes co-cultured with NK cells ranged between 5% and 62%. In four out of five donors TNF secretion was notably reduced when NKp80 was blocked **(Figure 2.24d, f** and **Table 2.4)**. These results indicate that NKp80 engagement also influences cell contact-dependent TNF secretion by monocytes. Finally, a contribution of NKp80 to monocyte-induced IFN-γ secretion by NK cells was also observed in a setting where experimental addition of monokines was substituted with LPS treatment of monocytes **(Figure 2.25)**.

	CD56^{bright} NK cells				
	NK-mono + IL				NK-mono
Donor	IgG1	5D12	7F12	5D12 7F12	–
1	14.1 ± 0.9	9.1 ± 2.2**	13.1 ± 1.5	9.4 ± 0.4*	1.6 ± 1.7
2	40.2 ± 2.8	25.5 ± 4.3*	ND	27.5 ± 4.8**	1.0 ± 0.4
3	4.4 ± 0.1	1.7 ± 0.3**	3.6 ± 0.5*	1.6 ± 0.3**	0.5 ± 0.2
4	9.7 ± 1.1	6.7 ± 0.9*	13.4 ± 2.3	7.0 ± 1.4	2.1 ± 0.7
5	3.8 ± 0.3	1.5 ± 0.3**	3.5 ± 0.7	1.7 ± 0.4**	0.9 ± 0.4
6	8.1 ± 0.9	ND	ND	5.5 ± 0.5*	0.3 ± 0.04
	CD56^{dim} NK cells				
	NK-mono + IL				NK-mono
Donor	IgG1	5D12	7F12	5D12 7F12	–
1	6.4 ± 0.6	4.8 ± 0.4*	6.2 ± 0.7	4.1 ± 0.2*	1.4 ± 0.1
2	25.2 ± 0.9	17.3 ± 2.6*	ND	18.6 ± 3.4*	1.2 ± 0.1
3	2.0 ± 0.1	1.1 ± 0.1**	1.8 ± 0.03	1.4 ± 0.1**	0.5 ± 0.1
4	3.0 ± 0.3	2.5 ± 0.2	3.0 ± 0.1	2.6 ± 0.2	0.6 ± 0.1
5	1.7 ± 0.2	1.2 ± 0.2*	1.6 ± 0.2	1.1 ± 0.2*	1.0 ± 0.2
6	11.1 ± 2.6	ND	ND	8.2 ± 1.1	2.1 ± 0.7
	CD14^{dim}CD16^{+} monocytes				
	NK-mono + IL				NK-mono
Donor	IgG1	5D12	7F12	5D12 7F12	–
1	51.7 ± 1.2	47.2 ± 2.2*	49.1 ± 1.8	46.8 ± 1.3*	14.7 ± 0.7
2	35.6 ± 1.4	28.9 ± 1.1*	ND	30.7 ± 0.6*	8.2 ± 0.7
3	13.8 ± 0.9	10.4 ± 0.6**	12.6 ± 0.7	10.0 ± 0.4**	1.9 ± 0.2
4	25.7 ± 0.5	22.3 ± 1.6	24.1 ± 1.5	21.4 ± 0.7**	10.7 ± 0.9
5	5.2 ± 0.5	4.0 ± 0.3*	4.6 ± 0.4	3.7 ± 0.4*	1.3 ± 0.2
6	61.9 ± 4.4	ND	ND	54.1 ± 1.5*	24.1 ± 4.8

Table 2.4. **Frequencies of IFNγ^{+} NK cells and TNFα^{+} CD14^{dim}CD16^{+} monocytes in NK-monocyte co-cultures.** Data represent percentages of IFNγ^{+} NK cells (columns 2-11) and TNFα^{+} CD14^{dim}CD16^{+} monocytes (columns 12-16) in 12h NK-monocyte co-cultures from six unrelated donors with monokines IL-15 and IL-18 (IL) or without monokines (columns 6, 11, 16). Significant reductions of frequencies in presence of 7F12, 5D12 or 7F12/5D12 as compared to control IgG1 (endotoxin-low (Fab')$_2$ –fragments were used throughout) are marked by: *, $p < 0.04$ or **, $p < 0.001$ (p-values were calculated using the two-tailed Student's t test). Data are means of n = 3 (Donor 1, 2, 4, 6) or n = 4 (Donor 3) or n = 6 (Donor 5) ± s.d. nd = not done.

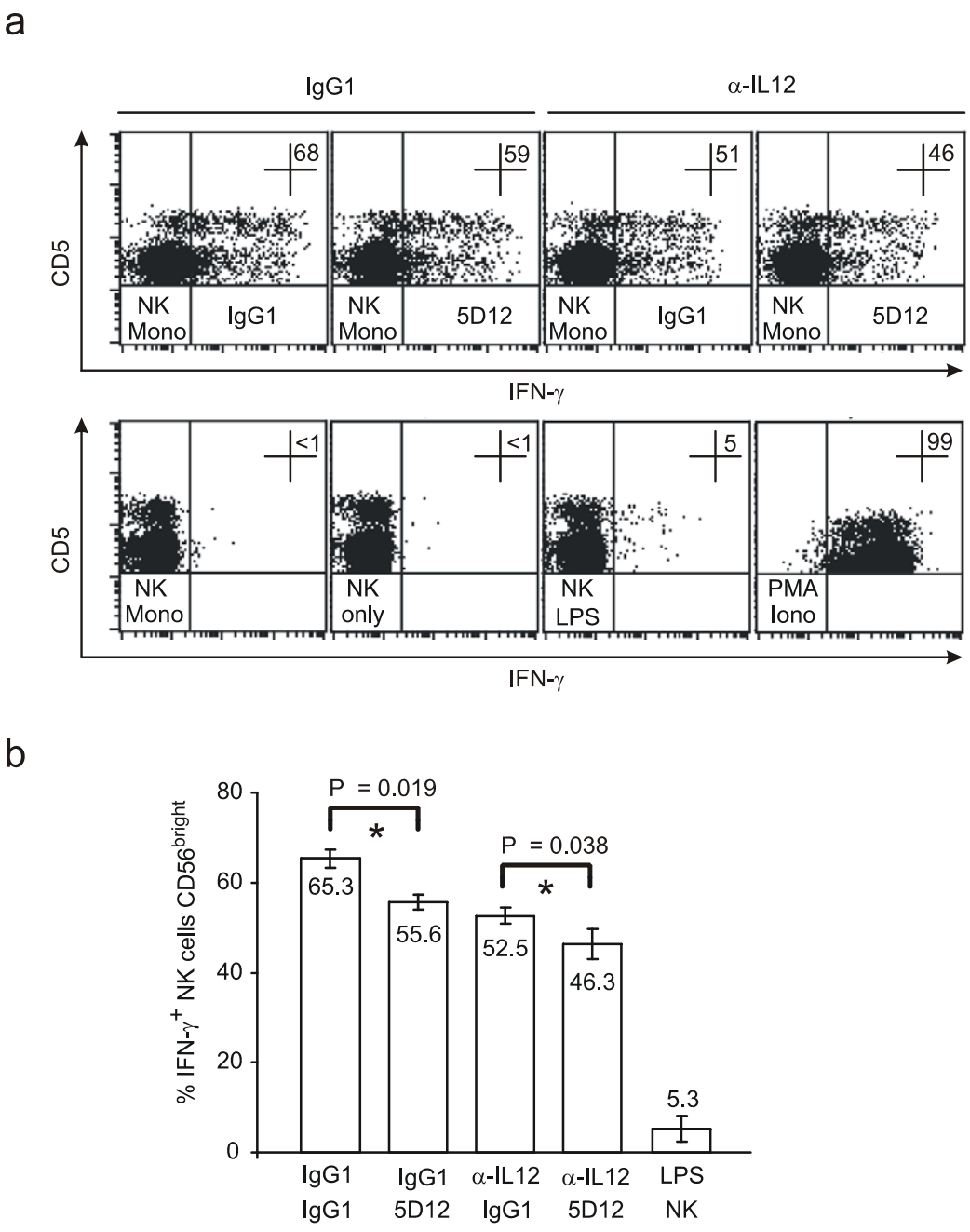

Figure 2.25. Frequencies of IFN-γ-producing NK cells after 12 h co-culture with autologous CD14^{dim}CD16^{+} monocytes in the presence of LPS. (a) Top, representative analysis of NK cells cultured with monocytes and 0.1 µg/ml LPS in the presence (right panels) or absence (left panels) of neutralizing anti-IL-12 (α-IL12) and in the presence of anti-NKp80 5D12 or an isotype control IgG_1. Bottom, various controls. Percentages of IFN-γ^{+} CD56^{bright} NK cells (upper right quadrant) are given. 5D12 and corresponding IgG_1 control were F(ab')$_2$ –fragments and all antibodies were used at 20 µg/ml. **(b)** Frequencies of IFN-γ^{+} CD56^{bright} NK cells after co-culture with monocytes in presence of LPS as described in **(a)**. All data are means of pentaplicates, errors bars represent s. d. *P*-values were calculated using the Mann-Whitney Rank Sum test and * indicates a significant difference. The experiment in **(b)** is representative of 2 independent experiments.

2.4.5 Discussion

NK cell activity is governed by the complex interplay of multiple activating, inhibitory and co-stimulatory receptors [**3**]. Hence, a thorough understanding of NK cell biology requires the functional definition of these various receptors and their ligands. Identification of MHC class I-related ligands and characterization of their stress-inducible regulation was key to understanding NKG2D function [**14**] and its role in immunosurveillance of viral infections and malignancies as well as in the pathogenesis of autoimmune diseases [**3,19**]. Like NKG2D, NKp80 is a homodimeric NKC-encoded activating NK receptor without a known inhibitory counterreceptor [**16,17**]. In contrast to NKG2D, NKp80 is predominantly expressed on NK cells and is absent in rodents. Hence, characterization of NKp80 ligands would likely aid the attempt to further decipher human NK cell biology.

We here identify AICL as a ligand of NKp80 and provide the first characterization of AICL as a myeloid-specific, activating receptor. AICL shares several features with TREM-1 including myeloid-specific expression, up-regulation in response to TLR stimulation and down-regulation during differentiation from monocytes to immature DCs [**32**]. In fact, AICL transcripts are among the most prominently down-regulated transcripts during *in vitro* generation of DCs from monocytes [**34**]. Like TREM-1, cross-linking of AICL triggered TNF release by monocytes, which was further enhanced by LPS stimulation [**32**]. However, unlike AICL, TREM-1 is a member of the immunoglobulin superfamily with yet unidentified ligands, is not expressed on $CD14^{dim}CD16^{+}$ monocytes and contains a positively charged amino acid in the transmembrane domain that allows pairing with the ITAM-bearing adaptor protein DAP12 [**32**]. The cytoplasmic domain of AICL is rather short (7 amino acids) and the transmembrane domain lacks charged residues, suggesting that AICL does not associate with DAP10, DAP12 or FcεRIγ adaptor proteins.

Recently, association of the distant AICL relative CD69 with sphingosine-1-phosphate receptor 1 has been reported [**35**]. In ongoing studies we will attempt to identify AICL-associated proteins and to assess a potential association between AICL and human sphingosine-1-phosphate receptors. Similarly, the signal transducing elements associated with NKp80 have not yet been defined. Moretta and colleagues originally reported that tyrosine phosphorylation of NKp80 was detected upon treatment of NK

cells with pervanadate, but immunoprecipitation experiments failed to identify NKp80-associated signaling molecules [**16**].

Certainly, it will be important to determine whether NKp80-AICL interactions influence NK cell reactivity and immune responses *in vivo*. Since both receptors have no corresponding sequence homologues in rodents, addressing this issue *in vivo* remains difficult. Here we provide *in vitro* data that can be conceptualized in two non-exclusive ways. First, we show that expression of AICL, which engages NKp80, increased susceptibility of myeloid cells to NK cell-mediated cytolysis. However, whereas malignant U937 cells were strongly lysed by NK cells, NK cell-mediated cytolysis of autologous LPS-activated monocytes was considerably lower or even absent depending on the donor. NK cell-mediated killing of infected monocytes and macrophages has been reported [**36**], and in these situations TLR-induced AICL expression may aid in the elimination of macrophages and other myeloid cells exposed to or infected by pathogens. Second, we demonstrated that secretion of pro-inflammatory cytokines in co-cultures of NK cells and monocytes in the presence of monokines was strongly augmented by NKp80 engagement, and that NKp80-AICL interactions account, at least in part, for the previously described cell contact-dependency of the activating cellular cross-talk [**12**]. Since this reciprocal activation involves multiple cytokines and possibly several receptor-ligand interactions, it is not unexpected that it was not completely blocked by anti-NKp80 treatment. Of the two AICL-specific antibodies generated, 7G4 does not block NKp80 binding, and 7F12 only partially inhibits NKp80 binding as judged from binding assays with recombinant proteins. This may account for the inefficient inhibition of cytokine production in NK-monocyte co-cultures by 7F12. However, our data do not exclude the possibility that a second unidentified NKp80-L on monocytes may also contribute to this cross-talk.

Our data establish the affinity of the NKp80-AICL interaction in the range of 2-5 μM. Affinities of other NKC-encoded homodimeric C-type lectin-like receptor-ligand pairs are not available for comparison. Reported affinities for NKC-encoded NK receptors interacting with MHC class I molecules or MHC class I-related molecules vary between 10 nM and 100 μM [**17**] (e.g. kinetic data of NKG2D-MICA interactions are similar to those of NKp80-AICL interactions [**37**]).

Yokoyama and colleagues were the first to describe the genetic linkage of certain receptor-ligand pairs within the NKC [**27**]. This observation aided the recent characterization of LLT1 as ligand of human CD161 [**28,29**], as well as the

identification of AICL as ligand of NKp80 presented here. In light of these findings, it is tempting to speculate that other adjacent genes (e.g. those encoding DCAL1 and CD69) located within this NKC subregion may encode receptor-ligand pairs. Whereas the NKC of mice contains genes encoding several inhibitory and activating Nkrp1 receptors, the single human Nkrp1 homologue NKRP1A is an inhibitory receptor [**17**]. Since Nkrp1 receptors and their Clr ligands in mice are clustered in a subregion of the NKC orthologous to the human NKC subregion encoding NKRP1A, LLT1, NKp80, and AICL, NKp80 may be a human equivalent of an activating mouse Nkrp1 receptor. Interestingly, transcripts for Clr-b and Clr-g, ligands of the inhibitory Nkrp1d and the activating Nkrp1f receptors, respectively, were reported to be expressed by myeloid cells [**27**]. However, lack of specific antibodies impeded detailed characterization of Clr protein expression. It has been proposed that the tight genetic linkage of Nkpr1-Clr receptor-ligand pairs reflects genetic strategies of ancient histocompatibility systems [**27**]. At least for humans, our analyses of AICL expression as well as our studies of LLT1 expression (J. Pfeiffer and A.S., unpublished observations) show that these ligands are specifically expressed by distinct subsets of hematopoietic cells, suggesting that these NKC-encoded receptor-ligand pairs may have evolved to orchestrate immune interactions between various leukocyte subpopulations.

In contrast to the activating receptor NKG2D, which is thought to alert NK cells towards ‘dangerous’ (e.g. infected or malignant) cells by detecting stress-induced self-ligands [**3,19**], NKp80 may mediate cell contact-dependent communication between NK cells and myeloid cells during early phases of infection or during chronic inflammatory reactions. Recent studies indicate that NK cells are activated by mycobacteria-infected monocytes and respond to Plasmodium-infected erythrocytes in concert with monocytes and macrophages [**11,38,39**]. Hence, addressing an involvement of the NKp80-AICL interaction in the immune control of these pathogens is of immediate interest.

In summary, here we identify the orphan AICL as a ligand of the human activating NK receptor NKp80 and characterize AICL as a myeloid-specific activating receptor (**Figure 2.26**). We provide evidence that NKp80 engagement by AICL not only promotes cytolysis of myeloid cells, but is also critically involved in the mutual activation of NK cells and monocytes.

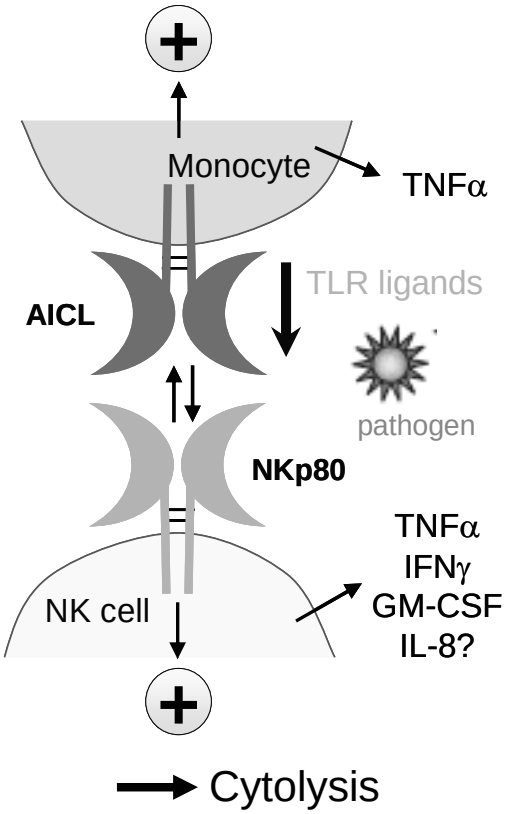

Figure 2.26. Activating cellular cross-talk between NK cells and monocytes is partially dependent on the NKp80-AICL interaction. AICL is a physiological ligand of the NK receptor NKp80. AICL is specifically expressed only on cells of the myeloid linage and is markedly induced by TLR ligands (e.g. LPS-containing pathogens). Cross-linking of AICL stimulated TNFα-release by monocytes and the NKp80-AICL interaction promoted lysis of AICL-expressing cells by NK cells. Finally resulted NKp80 blocking in a reduced sectretion of pro-inflammatory cytokines, i.e IFNγ from NK cells and TNFα from monocytes. Therefore the activating cellular cross-talk between NK cells and monocytes is partially dependent on the NKp80-AICL interaction.

To our knowledge, this is the first report describing a cellular cross-talk between human NK cells and monocytes mediated by cell-type specific receptors. Therefore these findings may provide insight into communication within the innate immune system in acute and chronic inflammatory situations, and may aid in the elucidation of processes of innate immune defense against human pathogens.

2.4.6 Acknowledgements

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3 Summary

The immunoreceptor NKG2D promotes immunosurveillance of malignant cells and protects the host from tumour initiation by activating NK cells and costimulating CD8 T cells. MICA and other ligands of NKG2D are frequently expressed by tumour cells. Human tumour cells are thought to avert NKG2D-mediated-immunosurveillance by shedding MICA and other NKG2D ligands (NKG2DL).

This thesis shows that the GPI-anchored NKG2DL ULBP2 is released from the cell surface of tumour cells by the action of metalloproteases similarly to the type I transmembrane proteins MICA and MICB. In addition, soluble ULBP2 was detected in the serum of patients with hematopoetic malignancies. Shedding of MICA and ULBP2 from tumours was induced by activation of protein kinase C (PKC) and was inhibited by the same compounds suggesting that ULBP2 and MIC molecules are released by the same or closely related proteases.

Further, the molecular mechanisms of MICA shedding were defined to characterise the proteases involved. Amino acid deletions in the membrane-proximal stalk region of the MICA ectodomain greatly impaired MICA shedding, whereas amino acid substitutions had no significant effect. Further, MICA shedding was blocked by specific inhibitors of "a disintegrin and metalloprotease" (ADAM) proteases and was markedly reduced when ADAM10 and/or ADAM17 were down-regulated by RNA-interference.

Altogether, these data demonstrate that ADAM10 and ADAM17 are critically involved in the proteolytic release of soluble MICA by tumours and thereby likely contribute to tumour immune evasion. Therefore, therapeutic blockade of ADAM10 and ADAM17 activities may represent a novel attractive approach to improve the efficacy of immunotherapeutic cancer treatment.

Zusammenfassung

NKG2D ist ein aktivierender NK-Zell Rezeptor und ein kostimulierender Rezeptor auf CD8 T Zellen. Die Expression der NKG2D Liganden (NKG2DL) wird durch Zellstress, virale Infektion und im Zuge maligner Transformation induziert und ermöglicht so dem Immunsystem die Erkennung und Elimination von veränderten und potentiell „gefährlichen“ Körperzellen (z. B. Tumoren). Die Freisetzung von löslichen NKG2D Liganden durch Metalloproteasen wird als ein wichtiger Mechanismus der Tumorzellen zur Vermeidung einer Immunantwort erachtet.

In dieser Arbeit konnte gezeigt werden, dass auch der NKG2DL ULBP2, wie z. B. die MIC-Moleküle MICA und MICB, in löslicher Form von der Zelloberfläche von Tumorzellen freigesetzt wird. Lösliches ULBP2 konnte im Serum von Leukämie-Patienten nachgewiesen werden. Die Tumor-assoziierte Freisetzung sowohl von ULBP2 als auch von MICA ließ sich durch Aktivierung der Protein Kinase C verstärken und durch die gleichen Inhibitoren blockieren. Dies führte zu der Schlussfolgerung, dass sowohl die MIC Moleküle als auch ULBP2, als Vertreter der GPI-verankerten ULBPs, durch die gleichen oder nah verwandte Proteasen freigesetzt werden.

Zur Identifizierung dieser Proteasen wurde beispielhaft der NKG2D Ligand MICA gewählt. Es konnte gezeigt werden, dass MICA in der Stielregion, die sich zwischen der Transmembranregion und der Ektodomäne befindet, gespalten wird. Wichtig für die Freisetzung ist nicht die Aminosäuresequenz, sondern die Länge dieser Stielregion. Des Weiteren konnte die Bildung von löslichem MICA durch Inhibitoren, die spezifisch gegen Mitglieder der ADAM (eine Disintegrin und Metalloprotease) Proteasen gerichtet sind, verringert werden. Die aufgrund dieser Experimente getätigte Annahme, dass ADAM10 und ADAM17 an der Freisetzung von löslichem MICA beteiligt sind, konnte in zwei unabhängigen Versuchsansätzen durch transiente Expressionssuppression dieser ADAMs in zwei Tumorzelllinien bestätigt werden.

Zusammenfassend konnte gezeigt werden, dass die Metalloproteasen ADAM10 und ADAM17 an der Freisetzung von löslichem MICA von Tumorzellen beteiligt sind und somit einen interessanten Ansatzpunkt zur Verbesserung der Immuntherapien von Tumoren bieten.

4 Abbreviations

Ab	antibody
ADAM	a disintegrin and metalloprotease
ADAM-TS	ADAM with trombospondin motif
ADCC	antibody dependent cellular cytotoxicity
AML	acute myeloid leukemia
APC	allophyocyanin
APP	amyloid precursor protein
ATM	ataxia telangiectasia, mutated
ATR	ATM and Rad3-related
BIM I	Bisindoylmaleimide I
BB94	Batimastat
BFA	Brefeldin A
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
CLSF	C-type lectin-like superfamily
CML	chronic myeloid leukemia
CTL	cytotoxic lymphocyte
CTLR	C-type lectin-like receptor
DC	dendritic cell
DAP10	DNAX activating protein of 10 kDa
DAP12	DNAX activating protein of 12 kDa
DMEM	Dulbecco's Modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular-signal regulated kinase
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluorescein 5-isothiocyanate
FoxM1	Forkhead Box M1
G418	neomycin
GPI	glycosylphosphatidylinositol
GM-CSF	granulocyte macrophage colony-stimulating factor
HCMV	human Cytomegalovirus
HLA	human leukocyte antigen
HB-EGF	heparin-binding epidermal growth factor
HRP	horse radish peroxidase
IFN	interferon
Ig	immunoglobulin
IGFBP	insulin-like growth factor-binding protein
IgSF	Ig superfamily
IL	interleukin
ILT	Ig-like transcripts
IMDM	Iscove's modified Dulbecco's medium

ITAM	tyrosine-based activation motif
ITIM	tyrosine-based inhibiton motif
kDa	kilo Dalton
KIR	Killer cell Ig-like receptor
LPS	lipopolysaccaride
LRC	leukocyte receptor complex
mAb	monoclonal antibody
MAdCAM	mucosal vascular addressin cell adhesion molecule
MALDI	matrix associated laser desorption/ionisation
MAP	mitogen activated kinase
MCA	methylchloanthrene
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIC(A/B)	MHC class I chain related protein A/B
sMICA	soluble MICA
sMICB	soluble MICB
MIP	monocyte chemotactic protein
MMP	matrix metalloprotease
MMPI	MMP inhibitor
MP	metalloprotease
mRNA	messenger RNA
MULT1	murine UL16-binding protein-like transcript 1
NCR	natural cytotoxicity receptor
NK	natural killer
NKC	natural killer gene complex
NKG2	natural killer group 2
NKG2DL	NKG2D ligand
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI3K	phosphatidylinositol 3-kinase
PI-PLC	phosphatidyinositol-specific phospholipase C
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PNGaseF	peptide:N-Glycanase F
RAG	recombination activating gene
Rae1	retinoic acid early inducible 1
RAET1	retinoic acid early inducible transcript 1
RANTES	regulated on activation, normal T cell expressed and secreted
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
SFI	specific fluorescence intensity
SH2	Src homology 2
SHP	SH2-containing protein tyrosine phosphatases
STAT	signal transducer and activator of transcription
sULBP	soluble ULBP
sAML	secondary AML
SVMP	snake venom metalloprotease
TACE	TNF-α converting enzyme
TBS	tris-buffered saline
TGF	transforming growth factor
TIMP	tissue inhibitors of metalloproteases

TLR	toll-like receptor
TNF	tumour necrosis factor
TNFRSF	TNF receptor superfamily
T-NHL	T-cell non-Hodgkin's-lymphoma
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation induced cytokine
ULBP	UL16-binding protein
UPN	unique patient number
UV	ultraviolet
VCAM	vascular cell adhesion molecule
ZAP70	zeta-chain-associated protein 70 kDa

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6 Publications

1. **Waldhauer I**, Steinle A (2006) Proteolytic release of soluble UL16-binding protein 2 from tumor cells. *Cancer Res* 66:2520-2526.

2. Eisele G, Wischhusen J, Mittelbronn M, Meyermann R, **Waldhauer I**, Steinle A, Weller M, Friese MA (2006) TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. *Brain* 129:2416-2425.

3. Welte S, Kuttruff S, **Waldhauer I**, Steinle A (2006) Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. *Nat Immunol* 7:1334-1342.

4. **Waldhauer I**, Göhlsdorf D, Gieseke F, Weinschenk T, Stevanovic S, Rammensee HG, Steinle A. Tumor-associated MICA is shed by ADAM proteases. (submitted)

5. **Waldhauer I**, Steinle A. NK cells and cancer. (Review, submitted)

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