Targeting MHC-I related proteins for cancer diagnosis and therapy

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Chapter 8:

Summary
General discussion
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Tumor-specific antigens

This thesis is focused on two antigens expressed on the surface of tumor cells, MICA, and HLA-E. Both antigens are members of the MHC-I family of proteins, and both act as ligands for the NK receptors on NK cells and $CD8^+$ T cells.

MICA

MHC-class I polypeptide-related sequence A (MICA), is often found overexpressed on the surface of cancer cells of hematopoietic and epithelial origin[227]. MICA is one of the ligands of NKG2D, an activating receptor found on NK cells and $CD8^+$ T cells, and gd T cells[218]. Engagement of NKG2D leads to their activation and triggers the cytotoxic activity of these immune cells. Because MICA is absent from the surface of healthy cells, we suspect that targeting this antigen to eliminate the cells that carry it should result in minimal harmful off-target effects.

While the typical immunoglobulins exert their effector functions through their Fc portion, their size compromises efficient tissue penetration. Intact immuno-globulins are less efficient at detecting their targets when using non-invasive imaging methods such as immuno-PET because of their comparatively poor tissue penetration and long circulatory half-life[371,409,413]. Nanobodies, also referred to as VHHs, are engineered from the variable regions of camelid-derived heavy-chain only antibodies. Nanobodies are characterized by their small size, allowing superior tissue penetration compared to full-sized antibodies. Nanobodies retain their antigen binding properties and are easier to produce and modify than conventional immunoglobulins. Furthermore, because of significant homology between human V_H regions and the V regions in VHHs[556] nanobodies are considered poorly immunogenic. Nanobodies thus offer an appealing alternative to immunoglobulins for the purpose of launching an immune attack on MICA-positive tumors.

Production and validation of MICA-targeting nanobodies

We produced nanobodies that recognize MICA by immunizing an alpaca with recombinant MICA*009, one of the most common alleles of MICA found in the Caucasian population. Plate-based panning of a phage library for binders yielded several nanobodies that recognize MICA*009 by ELISA. Based on sequence analysis, we chose clones that were unique in the CDR1, 2, and 3 regions (Chapter 3, Figure 1). Although sequence analysis and thorough

characterization of germline regions is impossible without access to the germline VH sequences of the source alpaca, with the help of literature reporting germline VH sequences of different alpacas, and by comparing the sequences of the obtained anti-MICA VHHs, we can hypothesize on the source of the anti-MICA nanobody sequences.

Based on sequence similarities found in the literature, we ascribe the alpaca IGHHV-3-3*01 germline gene to the D8, C12, and 2A9 nanobodies[556]. Somatic hypermutations occur mostly in the CDR regions, as the framework regions are generally less tolerant to such substitutions. VHH A1 and VHH B11 have a single L2V substitution in the framework region compared to the D8, C12, and 2A9 nanobodies. The A1 and B11 nanobodies could thus be derived from a different germline V gene, although neutral substitutions such as the L2V have been observed in framework regions as well[641]. VHH A1 and B11 may thus also be derived from the same germline V gene as VHHs D8, C12, and 2A9.

Despite the homology in framework regions 1 and 2 of VHH 2B5, this nanobody has two non-neutral substitutions in framework region 3 compared to the consensus sequence of all the anti-MICA nanobodies. The R72S and N74I substitutions imply that VHH 2B5 is likely derived from a different germline V region. Nanobody E9 also has two substitutions, a neutral D29E in framework region 1 and a basic-to-polar R45Q in framework region 2. VHH E9 is thus likely derived from a different V gene. Nanobody 2D5 has a polar to non-polar S49A substitution in framework region 2 and is likely derived from a different germline V gene. Nanobody H3 shows the largest number of variations and based on alpaca germline sequences described in the literature, we hypothesize that this nanobody is derived from the alpaca IGHHV3-1*01 V gene[556].

The CDR2 regions of VHH C12, 2A9, B11, E9, and 2D5 show a deletion at position 53. Although somatic hypermutation can produce deletions and insertions in V genes[566–568], given the overall dissimilarity in framework regions, the use of distinct V genes that lacks residue 53 is the more plausible explanation. Similar CDR regions, specifically CDR3, imply recognition of related antigens[562–565]. Except for H3, A1 and 2B5, the remaining CDR3 regions are enriched for the sequence “AxDCLSSxWRx”.

To select which nanobodies to use for downstream applications, we performed an ELISA to estimate affinities, and a competition assay to determine whether the different VHHs recognize distinct epitopes on MICA. Based on those results, we chose VHH-A1 and VHH-H3 for downstream applications, because they bind to recombinant MICA*009 with high affinity

(~0.2 nM and ~0.4 nM respectively) (Chapter 3, figure 2C). The results of the competition ELISA suggest that these nanobodies can be used in synergy, since they seem to recognize different epitopes on the protein (Chapter 3, Figure 2A). Both VHH-A1 and VHH-H3 recognize the alleles MICA*008 and MICA*009 (Chapter 3, Figure 2D), which together cover >50% of the Caucasian population[557]. To our knowledge, these are the first examples of nanobodies specific to MICA.

These nanobodies can be modified at the C-terminus in a sortase-catalyzed reaction because of the presence of a C-terminal LPETG sortase recognition motif. This enables the addition of biotin, fluorophores, other molecules, and even intact proteins[553,635]. By biotinylating the nanobodies and using a streptavidin-conjugated horse radish peroxidase (HRP) or fluorophore as detection agent, we show that VHH-A1 and VHH-H3 recognize immobilized or denatured recombinant MICA by immunoblot and ELISA (Chapter 3, Figure 2B and 2D). More importantly, we show that these nanobodies recognize surface-disposed MICA on MICA-transfected B16F10 and EL-4 cancer cells, assessed by flow cytometry (Chapter 3, Figure 2E). B16F10 and EL-4 cells are murine cells, derived from a melanoma and T-cell lymphoma respectively. Because mice do not express MICA/B or proteins that show cross-reactivity with anti-human MICA/B reagents, we could use the MICA transfectants of these cancer cell lines to apply the nanobodies *in vivo*.

MICA-targeting nanobodies can be used to generate nanobody-drug conjugates

Maytansines DM1 and DM4 are small molecules that disrupt microtubules. Adducts of antibodies with Maytansine have been approved for clinical use for the treatment of $HER2^+$ breast cancer (ado-trastuzumab emtansine[642]) and folate receptor alpha positive, platinum-resistant epithelial ovarian, fallopian tube, or primary peritoneal cancer (Mirvetuximab soravtansine-gynx[643]).

We created a nanobody-drug conjugate (VHH-A1-DM1) by conjugating a Maytansine derivative DM1 to VHH-A1 in a sortase-mediated reaction (Chapter 3, Figure 3A and 3B). We observed decreased proliferation in EL-4 $MICA^+$ tumor cells treated with VHH-A1-DM1 compared to EL-4 WT tumor cells *in vitro*. The calculated IC_{50} is comparable to that of free Maytansine (DM4) (Chapter 3, Figure 3C and 3D). The difference between DM1 and DM4 is the inclusion of an additional dimethyl group next to the terminal cysteine in DM4, which increases the hydrophobicity and facilitates cell penetration of

DM4, thus increasing the cytotoxic effects[644]. In our experiments, an adduct of VHH-A1-DM4 proved ineffective in killing MICA$^+$ cells (Chapter 4, Figure 1C). Perhaps the cleavable linker between VHH-A1 and DM4 releases the cytotoxic payload into the medium and decreases the intracellular cytotoxicity.

The VHH-A1-based nanobody-drug conjugate was ineffective in treating B16F10-derived MICA$^+$ cells *in vitro* (Chapter 4, Figure 1B). Although B16F10 has been reported more resistant to treatment with DM1 alone, the precise reasons for this resistance remain to be identified[578]. Inclusion of cell lines that represent different tumor types will be required to determine the extent of resistance to VHH-A1-DM1.

For our *in vivo* model, we extended the half-life of the nanobody drug conjugate by creating a genetic C-C fusion of VHH-A1 to an anti-murine kappa light chain nanobody (VHH-mKappa). We ligated DM1 to this fusion by sortase reaction to create VHH-A1-VHH-mKappa-DM1 (Chapter 4, Figure 2A). Mice bearing subcutaneous EL-4 MICA$^+$ cells were treated 3x weekly with an intraperitoneal injection of 5 mg/kg of this fusion (Chapter 4, Figure 3A). We showed that the VHH-A1-VHH-mKappa-DM1 was ineffective in treating EL-4 MICA$^+$ tumors (Chapter 4, Figure 3B and 3C). Although tumor growth in the treated mice started slower compared to mice treated with a non-targeting nanobody-drug conjugate, once treatment was stopped this difference disappeared. In fact, the treated mice showed accelerated tumor growth upon cessation of treatment. Perhaps intravenous administration of VHH-A1-DM1 improves its delivery to the tumor. Further research is needed to validate the efficacy of VHH-A1-DM1 *in vivo.* Extending the half-life of the nanobody-drug conjugate is important for its persistence *in vivo* and might instead be achieved by conjugation of the nanobody-drug conjugate to polyethylene glycol (PEG20).

The creation of different VHH-drug combinations, for example using other tubulin inhibitors like Auristatins, DNA damaging agents like Exatecans, immuno-modulators like STING agonists, or radiopharmaceuticals for targeted radiotherapy, deserves consideration. We have not performed a direct comparison of the nanobody-drug conjugated to other MICA/MICB targeting agents, such as full-sized monoclonal antibodies. This could be done by engineering the coding sequence(s) for such reagents to contain a

sortase motif for site-specific conjugation, as was done in this thesis for the HLA-E cytoplasmic tail-specific monoclonal antibody.

MICA-targeting nanobodies can be used in VHH-based CAR NK cell therapy

A relatively new, now widely explored form of immunotherapy is adoptive cell transfer, and more specifically, the use of T and NK cells modified with a chimeric antigen receptor (CAR). scFv-based CAR T cell therapy is already considered as a possible cancer therapy, with several such therapies approved by the FDA for treatment of relapsed or refractory multiple myeloma and B-cell lymphoma, based on BCMA and CD19 targeting respectively. However, some limitations of scFv-based CARs merit discussion. First, scFvs used as targeting moieties may lack the affinity and specificity of the immunoglobulin from which they are derived. Furthermore, the immunogenicity of the scFv, as well as the potential of self-aggregation of scFv-based CARs, must be considered. This is relatively easy to diagnose and fix, for instance by grafting the CDR regions of the mouse-derived antibodies into human-derived framework region backbones[645]. In contrast, the single domain nature of VHHs precludes self-association, while VHHs retain excellent antigen recognition with binding constants typically in the nanomolar range. The clinical success of a nanobody-based CAR T cell, Carvykti, for the treatment of multiple myeloma is the first example of a clinically efficacious nanobody-based CAR T cell. This CAR contains a bi-paratopic ectodomain that recognizes the antigen BCMA, a protein highly expressed on fully mature plasma cells and on multiple myeloma[602]. The success of Carvykti establishes the suitability of nanobodies as building blocks for the construction of CARs targeting other antigens.

Although CAR T cell therapy is widely studied in the clinic, there are several possible advantages of using CAR NK cells instead. First, NK cells are easier to source and expand *ex vivo* than T cells, with the ability of producing CAR NK cells not only from the patient's or a donor's peripheral blood, but also from umbilical cord blood, manufactured from iPSCs, or from existing immortalized NK cell lines (e.g. NK-92). Unlike T cells, NK cells do not pose the risk of graft-versus-host disease (GVHD) in an allogeneic setting and are not MHC-restricted. CARs to be installed on NK cells allow the inclusion of a wider range of co-stimulatory domains such as CD244, CD137, and NK activating receptors. Furthermore, NK cells have an inherent innate anti-tumor response. Even if a tumor were to downregulate the CAR target, CAR

NK cells might still exert a cytotoxic effect. Lastly, NK cells release the cytokines IL-3, TNF-α, and IFN-γ, only the latter of which is associated with cytokine release syndrome, a major side-effect of CAR T therapy caused by excessive secretion of IL-2, IL-6, and IFN-γ. For these reasons, we opted for the production of CAR NK cells instead of CAR T cells.

We developed VHH-based CAR NK cells by lentiviral transduction of NK-92 cells with a construct containing VHH-A1 or VHH-H3 as targeting moiety, followed by a transmembrane domain and the intracellular CD3ζ co-stimulatory domain and the CD28 signaling domain (Chapter 5, Figure 1A). Successfully transduced cells were sorted based on GFP expression and expanded to create the stable A1 CAR NK, H3 CAR NK, or non-targeting empty vector (EV) CAR NK lines (Chapter 5, Figure 1B and 1C). We determined the efficacy of the CAR NK cells *in vitro* by co-culturing them with B16F10 melanoma cells or EL-4 T-cell lymphoma cells, and their MICA$^+$ transfectants. By measure of LDH-release, we observed a significant increase in cytotoxicity of MICA$^+$ B16F10 or MICA$^+$ EL-4 cancer cells when co-cultured with VHH-A1-based CAR NK cells, compared to WT B16F10 or EL-4 cells co-cultured with A1 CAR NK cells, or either line co-cultured with the EV CAR NK cells (Chapter 5, Figure 2A). This cytotoxicity is caused by activation of the A1 and H3 CAR NK cells, confirmed by a significant increase in IFNγ released by the A1 and H3 CAR NK cells co-cultured with MICA$^+$ B16F10 or MICA$^+$ EL-4 cells, compared to these cells co-cultured with WT B16F10 and EL-4 cells (Chapter 5, Figure 2B).

In vivo, using an ^{89}Zr-labeled nanobody that targets the transferrin receptor on the NK-92 cells, we were able to track and localize the A1 CAR NK cells to MICA$^+$ lung metastases in mice by immuno-PET imaging. We observed a PET signal, and thus localization, to the lungs of mice bearing MICA$^+$ lung metastases until 72-hours after injection of A1 CAR NK cells. Less positive signal was observed in the lungs of mice bearing MICA$^+$ lung metastases which received EV CAR NK cells (Chapter 5, Figure 4B). We conclude that the A1 CAR NK cells, by virtue of finding and binding to their MICA$^+$ target cells, can persist longer *in vivo* than non-targeting CAR NK cells. This data provides us with insight into the parameters for administration of the VHH-based CAR NK cells for treatment of MICA$^+$ tumors.

In an *in vivo* model of mice grafted with primary subcutaneous B16F10 MICA$^+$ tumors, the A1 CAR NK cells are cytotoxic towards the MICA$^+$ B16F10 tumors,

as shown by a significant reduction in the rate of tumor growth and an increase in overall survival for the treated mice compared to mice treated with EV CAR NK cells (Chapter 5, Figure 3B). These findings suggest a therapeutic potential for the VHH-based CAR NK cells. It is important to note the low group size of n = 7 for the A1 CAR NK treated group and n = 3 for the EV CAR NK treated group. During the first treatment injection, we injected $10*10^6$ cells retro-orbitally, after which a third of the mice died. We suspect that the high number of cells injected at once created a blockage in the lung capillaries. Although the obtained results are significant, larger cohort sizes might increase our power. Given the PET imaging data with B16F10 metastasis model, it would be interesting to test the effect of treatment with the CAR NK cells on metastases formation.

MICA-targeting nanobodies to produce CAR T cells

Despite the potential benefits of CAR NK cell therapy over CAR T cell therapy, much work has already been conducted using CAR T cells and as mentioned, a nanobody-based CAR T cell therapy has been approved by the FDA. It would thus be interesting to pursue anti-MICA immunotherapy with CAR T cells. To this end, we produced VHH-based CAR T cells from murine primary T cells by retroviral transduction, using an MSCV-based vector. The CAR construct follows the same second-generation principal as the CAR NK cells, containing a GFP fluorophore followed by a P2A cleavage domain, VHH-A1 or VHH-H3 as targeting moiety, a transmembrane domain, and the intracellular CD3ζ co-stimulatory domain and the CD28 signaling domain (Chapter 6, Figure 1A and 1B). We were able to reach transduction efficiencies of ~35%, based on GFP expression (Chapter 6, Figure 1C). When using these CAR T cells in co-culture experiments, we observed significant activation and cytotoxicity in co-culture of A1 CAR T cells with $MICA^+$ B16F10 and $MICA^+$ EL-4 cells (Chapter 6, Figure 2A and 2B). However, these effects were only measured at high effector-to-target ratios of [1:10] or more. Because of the relatively low transduction efficiency and considering that only ~20% of the isolated murine T cell population consists of $CD8^+$ T cells (the population responsible for cytotoxicity), we more accurately have a VHH-based $CD8^+$ CAR T cell pool of ~7%. When mixing the effector and target cells at a ratio of [1:10], we have an effective [E:T] of 1:0.3, which is likely insufficient to create significant anti-tumor responses. To increase the [E:T], we could increase the number of T cells in the well. We hypothesize that this will overcrowd the wells and reduce viability of the CAR T cells, because T cells have an optimal density of 1 – $2.5x10^5$ cells/mL. To obtain more favorable [E:T] ratios without overcrowding the wells, we could decrease the amount of target cells.

Unfortunately, the assays we use currently for measuring cytotoxicity and IFN-γ release might not be appropriate for such low cell counts. We would thus have to find a more sensitive assay for measuring these parameters.

Due to the limitations in transduction efficiency of the CAR T cells, we did not pursue their *in vivo* properties. This remains an interesting avenue to pursue in future research since data from our lab and others has shown great potential of treating tumors with nanobody-based CAR T cells[472,476,550].

Future perspectives

For the present work, we created a second-generation CAR construct, utilizing co-stimulation and signaling by the CD28 and CD3ζ domains. To improve stability, activation, and signaling of these CAR NK cells, improvements to the CAR construct might include addition of the 4-1BB signaling domain, known to enhance persistence of the CAR NK cells *in vivo*. Further enhancement could be reached by including a cytokine auto-stimulation ectodomain, such as IL-15 for the CAR NK cells[611] or IL-2 for the CAR T cells[646], inclusion of additional co-stimulatory domains such as CD27 or STAT3/5 binding motifs[647,648], enhancement of the CD28 signaling domains by incorporation of certain null mutations of the CD28 subdomains[580,649] or the inclusion of immunoreceptor tyrosine-based activation motif (ITAMs) 2 and 3 in the CD3ζ portion[650].

The tumor microenvironment often shows increased deposition of extracellular matrix (ECM) components, which can cause encapsulation of a solid tumor and thus impose a physical barrier that limits access to the tumor for CAR T or CAR NK cells. CAR macrophages may help degrade the ECM by secretion of proteases, which improves the outcome of immunotherapy. Since we produced two nanobodies, H3 and A1, each recognizing a unique epitope on MICA, H3-based CAR macrophages might be able to help degrade the ECM and attract A1-based CAR T or CAR NK cells to aid in tumor-specific cytotoxicity.

For clinical translation of this research, several points are worth mentioning. Although MICA expression is typically absent from healthy cells, expression of MICA and MICB is seen in gut epithelium and could elicit a harmful 'off-tumor on-target' response[604]. Since gut epithelia are capable of rapid repair, this risk may prove to be manageable. Since mice do not possess a MICA/B homolog, this research should be extended to MICA$^+$ animals such as non-human primates, which express MICA/B type molecules. The use of a MICA-

transgenic mouse harboring the correct allele of MICA could provide a useful alternative to the often highly regulated research in non-human primates[606].

The genetic instability of NK-92 cells requires their irradiation prior to infusion in a patient to avoid the possibility of any malignant outgrowths of the NK population. However, irradiation impairs proliferation of the transferred NK-92 cells and thus limits their persistence *in vivo*. As an alternative, CAR NK cells could be created from patient-derived peripheral NK cells, although this method comes with its own limitations. NK cells only comprise 10% of circulating white blood cells, which requires *ex vivo* expansion on a cell line feeder layer, again incurring a risk[651]. In addition, allogeneic NK cells sourced from healthy donors or umbilical cord blood also require expansion, and T cells need to be carefully removed to avoid GVHD. Other alternative NK cell sources are induced pluripotent stem cell-derived NK cells (iPSC-NKs), which can easily be produced from a standardized, homogeneous cell population and grown to clinical scale.

We recognize the limitations of using cell lines in which MICA is expressed through transfection. We thus can't conclude on the efficacy of the nanobodies on human cancer cell lines naturally expressing MICA. The limiting factor for us here was the availability of patient-derived cell lines expressing the correct alleles of MICA. We think these are excellent avenues to explore in future research.

In cancer patients, MICA is often shed from the cancer cell surface, rendering the cells invisible to MICA-targeted immune attack. Shedding occurs when the α3 domain of MICA undergoes ECM-induced proteolytic cleavage, facilitated by the disulphide isomerase ERp5 and ADAM-type proteases such as ADAM10 and ADAM17[232–236]. Wucherpfennig and coworkers have generated a monoclonal antibody, 7C6, that inhibits shedding of MICA/B and thus increases the density of MICA/B proteins on the surface of tumor cells[243]. Combination therapy of MICA-targeting nanobody-drug conjugates or nanobody-based CAR NK or T cells with the 7C6 antibody may therefore be worth exploring to enhance the efficacy of treatment.

HLA-E

Conventional MHC-I molecules (HLA-A, -B, and -C) present peptides derived mostly from intracellular antigens. In humans, HLA-E is specialized in the presentation of peptides derived from the signal sequences of other MHC-I products, as well as the signal sequences of certain viral glycoproteins. Tumors often downregulate the surface expression of the classical MHC-I molecules encoded by the HLA-A, -B,and -C loci, thereby evading cytotoxicity exerted by $CD8^+$ T cells. In contrast, many cancer cells overexpress HLA-E. HLA-E acts, among other things, as a ligand of NKG2A, the inactivating or inhibitory receptor found on $CD56^{hi}$ NK cells and on a subset of $CD8^+$ T cells. Engagement of HLA-E by NKG2A inhibits the cytotoxicity of $CD56^{hi}$ NK and $CD8^+$ T cells, and thus can lead to immune evasion by the tumor[652].

Commercially available antibodies against the ectodomain of HLA-E, MEM-E/02 and 3-D12, display varying degrees of cross-reactivity with allelic products of the HLA-B and HLA-C loci[653]. A comparison of the sequences of HLA-E with those of other MHC-I molecules shows strong sequence conservation in the ectodomains. In contrast, the amino acid sequence of the cytoplasmic tail of HLA-E appears to be strongly conserved and distinct from the cytoplasmic tail sequences of the HLA-A, -B, and -C proteins (Chapter 7, Figure 1).

Peptide-bound HLA-E rapidly exits the endoplasmic reticulum (ER), traverses the secretory pathway, and is expressed at the cell surface. The cytoplasmic tail of MHC-I molecules plays a role in export from the ER and in endocytosis[280]. Most HLA-E, however, appears to be retained in an immature state in the ER, and it has been confirmed that the cytoplasmic tail of HLA-E plays a role in its ER retention and endocytosis[281].

Based on these observations, we developed a monoclonal antibody directed to the cytoplasmic tail of HLA-E. Because of the unique sequence of the cytoplasmic tail, we expect no cross-reactivity with other MHC-I molecules. This antibody can thus be used for diagnosis of HLA-E positive cancers. Furthermore, antibodies against the HLA-E cytoplasmic tail could be a useful tool for studying the cytoplasmic tail interactions.

A monoclonal antibody targeting the cytoplasmic tail of HLA-E

To target the cytoplasmic tail of HLA-E, we fused the peptide sequence of the cytoplasmic tail (GGCSKAEWSDSAQGSESHSL, referred to hereafter as

HLA-E_{tail}) to a murine MHC-II-targeting nanobody (VHH_{MHCII}) by a sortase-mediated reaction (Chapter 7, Figure 2A). Based on previous research, immunization of mice with this antigen-fused nanobody results in targeted delivery of antigen to antigen presenting cells and elicits strong B and T cell immunity[420,463]. We immunized C57/Bl6 mice with VHH_{MHCII}-$HLAE_{tail}$ and selected a mouse that showed a high titer for hybridoma production (Chapter 7, Figure 2B). To obtain hybridomas that target the extracellular tail peptide, and not VHH_{MHCII}, we performed all screening ELISAs on a fusion of GFP with $HLAE_{tail}$, obtained via sortase reaction. The mice were never exposed to GFP and should thus have no antibodies to this protein. So, any response on the ELISA plate would ensure specificity for the cytoplasmic tail peptide (Chapter 7, Figure 2C). We obtained several hybridomas, all of which used the identical heavy chain sequence derived from the VH IGHV1-72*01 and J IGHJ2*01 genes. The hybridomas used 3 VJ kappa light chain rearrangements, based on the usage of the germline Vκ IGKV1-135*01, IGKV4-90*01, and IGKV-50*01 genes (Chapter 7, Figure 3A and 3B)

By performing an immunoblot with the antibodies on GFP ligated to a series of overlapping peptides with a 1-residue pitch, we determined that the antibodies recognize a 7-residue epitope ("SAQGSES") (Chapter 7, Figure 5A). This sequence alone was insufficient as an epitope tag in combination with the antibodies. To determine the smallest possible tag, we expanded the sequence and created an 8-mer, 10-mer, and 13-mer peptide which we incorporated at the C-terminus of an unrelated protein. We overexpressed this protein in HEK-293T cells by transfection and subjected cell lysates to immunoblot with conditioned medium from hybridoma cultures. The monoclonal antibodies "19-H12" and "2-D12" recognized the 13-mer (WSDSAQGSESHSL) at the C-terminus of the target protein, but not the 8-mer or 10-mer (Chapter 7, Figure 5B). We ran a search of the sequence against all available protein sequences and found a hit only for HLA-E in humans, and its non-human primate homologs. Because this sequence is located in the cytoplasm, the use of the WSDSAQGSESHSL-tag in extracellular proteins in cells of human or non-human primate origin would be possible.

To explore whether recognition of the 13-mer tag by the antibodies is sequence context-dependent, we placed the 13-mer peptide sequence at the N- or C-terminus, or at an internal location of UBE2V2 and confirmed

immunoreactivity with both the 19-H12 and 2-D12 antibodies, independent of the location of the tag, by immunoblot (Chapter 7, Figure 5C). Immunoprecipitation further validated the interaction of the 19-H12 mAb with the 13-mer tag. The immunoprecipitated protein complex can be eluted by addition of an excess of free synthetic peptide (Chapter 7, Figure 5D, 5E, and 5F). Given the strong reactivity of the antibody in immunoblots, and the lack of cross-reactivity with endogenous proteins, WSDSAQGSESHSL may thus have utility as an epitope tag.

Site-directed modification of the monoclonal antibody

We modified the C-termini of the 19-H12 heavy and light chains with an LPETG sortase recognition motif by cloning a GBlock™ into a mammalian expression vector and producing the hybridomas in EXPI 293 cells. The addition of the LPETG motif allows modification of the antibodies by sortase-mediated transpeptidation reaction (Chapter 7, Figure 4). This method, when compared to more conventional methods of labeling antibodies, ensures reproducibility, site-specificity, and produces the desired product in excellent yield. Site-directed modification with fluorophores or biotin eliminates the need for secondary antibodies for detection in assays like flow cytometry. We have shown the functionality of the HLA-E_{tail} specific mAb for cell staining in immunoblot, immunofluorescence, flow cytometry, and immunohistochemistry (Chapter 7, Figure 6 and Figure 7).

Future perspectives

In conclusion, we have developed the monoclonal antibody 19-H12 which, in combination with its 13-residue epitope, can be used as epitope tag for extracellular proteins, since the 13-residue peptide is not found in any other protein except HLA-E. The epitope tag could be further explored for detection or purification of, for instance, a poorly immunogenic protein, or other proteins in a setting where the set of available epitope tags in current use is exhausted. The epitope mapping revealed a core epitope of 7 amino acids long ("SAQGSES"). We had to extend this sequence to the 13-mer, which we did by inclusion of the flanking amino acids present in the cytoplasmic tail peptide. Perhaps the 7-mer is the smallest epitope recognized by the antibody, and the 5-residue extension facilitates binding by aiding the 3D confirmation. To investigate this, we could flank the SAQGSES core epitope with unrelated amino acids and determine binding of the antibody.

The monoclonal antibody 19-H12, either directly labeled with biotin or fluorophores, or by using a secondary antibody, targets the cytoplasmic tail of HLA-E intracellularly as assessed by flow cytometry, immunofluorescence, and immunohistochemistry. Because of its epitope, the antibody will likely not cross-react with other MHC-I molecules.

As shown with immunohistochemistry, we can detect aberrant HLA-E expression on samples of human progressive non-muscle invasive bladder cancer at high sensitivity compared to MEM-E/02. These characteristics make 19-H12 a potential staple for diagnosis of HLA-E$^+$ tumors in the clinic.

Furthermore, studies on the involvement of the cytoplasmic tail in HLA-E trafficking through the endoplasmic reticulum and turnover from the cell membrane might benefit from this newly developed reagent. Its use does not require genetic modification of the target recognized and could thus find application in samples or cell lines established from primary tumors. Monoclonal antibody 19-H12, when labeled, can be used for detection of HLA-E intracellularly. Although not pursued in the context of this thesis, the ability to retrieve otherwise unmodified HLA-E molecules in pulse chase experiments might add further refinement to the study of intracellular trafficking of HLA-E. Understanding the transport pathways of HLA-E is essential for further elucidating HLA-E-restricted CD8$^+$ T cell responses, like those seen in the more recently developed cytomegalovirus (CMV)-based vaccines against SIV[286].