Aus dem Sonderforschungsbereich 650 („Zelluläre Ansätze zur Suppression unerwünschter Immunreaktionen - from bench to bedside“)
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

NK cells in patients undergoing allogeneic stem cell transplantation are influenced by the CMV UL40 gene polymorphism

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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aus Leipzig

Datum der Promotion: 23.06.2019

Contents

Abstract/Zusammenfassung

Natural Killer cells are large granular lymphocytes which take an important role in the immune control of cells with viral infection, mutations or cells coated with antibodies. Traditionally NK cells were believed to be part of the innate immune system. Over the last years, however, it has been shown that NK cells possess adaptive features as well. In humans NK cells with those adaptive features have been described in persons with a positive CMV-serostatus. Furthermore NK cells play an important role in patients undergoing allogeneic stem cell transplantation, most importantly for the control of infections and protection from relapse. With CMV and NK cells both playing an important in allogenic stem cell transplantation, this model seems to be perfect to study the impact of CMV on the generation of adaptive NK cells as well as the clinical impact of adaptive NK cells.

We studied the NK cell-CMV interaction with the use of adaptive NK cells derived from CMV-seropositive blood donors and in patients with CMV reactivation after allogeneic stem cell transplantation. Adaptive NK cells derived from CMV-seropositive donors showed a specific tuning of ex vivo effector functions by virus-derived peptides. By sequencing of the CMV UL40 gene in patients with CMV reactivation we could derive the respective peptide encoded for during CMV reactivation. In those patients with CMV-reactivation we were able to show that the generation of certain NK cell populations expressing the NKG2C-receptor is influenced by the CMV-peptide polymorphism. For example the change of one amino acid (VMAPRTL**I**L to VMAPRTL**F**L) was able to trigger a difference in the generation of NK cell populations with FACS-defined cell profiles.

Adaptive NK cells have strong effector functions and are thereby be thought to have anti-leukemia functions as well. The link of adaptive NK cells to a better clinical outcome after allogeneic stem cell has been suggested by some investigators. Even though our data, because of the small sample size, just give a hint into this direction, the therapeutic use of adaptive NK cells in patients with hematologic malignancy could certainly play an important role in the future.

Natürliche Killerzellen sind große granuläre Lymphozyten welche eine wichtige Rolle in der Immunantwort auf Zellen mit Virusinfektion, Mutationen oder antikörper-bedeckte Zellen besitzen. Typischerweise werden NK-Zellen dem angeborenen Immunsystem zugeschrieben. In den letzten Jahren hat sich allerdings herausgestellt, dass NK-Zellen auch adaptive Eigenschaften besitzen. In Menschen sind adaptive NK-Zellen vor allen Dingen in Personen mit positivem CMV-Serostatus beschrieben worden. NK-Zellen spielen außerdem eine wichtige Rolle in Patienten welche eine allogene Stammzelltransplantation erhalten, vor allem zum Schutz vor Infektionen und einem Rezidiv der Grunderkrankung. Da sowohl CMV als auch NK-Zellen eine wichtige Rolle in der allogenen Stammzelltransplantation spielen, eignet sich diese als gutes Modell zur Untersuchung des Einflussen von CMV auf die Generation von adaptiven NK-Zellen, als auch zur Untersuchung der klinischen Relevanz adaptiver NK-Zellen.

Wir haben die NK-Zell-CMV-Interaktion mithilfe von adaptiven NK-Zellen von gesunden CMV-seropostivien Blutspendern und innerhalb von Patienten mit CMV-Reaktivierung nach Stammzelltransplantation untersucht. Adaptive NK-Zellen von CMV-seropositiven Spendern zeigten dabei eine präzise Regulierung ihrer Effektor-Aktivitäten durch spezifische Virus-Peptide. Durch die Sequenzierung des CMV UL40-Gens in Patienten mit CMV-Reaktivierung konnten wir auf das jeweilig enkodierte Peptid während CMV-Reaktivierung rückschließen. In dieser Patientenkohorte mit CMV-Reaktivierung konnten wir zeigen, dass die Generation spezifischer NKG2C-positiver NK-Zell-Populationen durch den CMV-Polymorphismus beeinflusst wird. Zum Beispiel konnte der Austausch von nur einer Aminosäure (VMAPRTL**I**L zu VMAPRTL**F**L) eine unterschiedliche Generation von NK-Zell-Populationen, definiert durch das durchflusszytometrische Zell-Profil, verursachen.

Adaptive NK-Zellen haben starke Effektor-Funktionen, weswegen ihnen auch anti-Leukämie-Effekte zugeschrieben werden. Die Korrelation von adaptiven NK-Zellen mit klinischen Variablen nach allogener Stammzelltransplantation ist von verschiedenen Autoren beschrieben worden. Auch wenn unsere Daten, vor allem aufgrund der kleinen Probenzahl, nur einen kleinen Hinweis in diese Richtung geben, könnte die therapeutische Nutzung von adaptiven NK-Zellen in Patienten mit hämatologischer Grunderkrankung in Zukunft sicherlich eine wichtige Rolle spielen.

List of Figures and Tables

1 Introduction

1.1 NK cells

Natural Killer cells are large granular lymphocytes which are important for the detection and control of cells with viral infections, spontaneous mutations[1] and cells coated with antibodies[2]. They possess a sophisticated system of activation and inhibitory receptors, which specifically recognize changes on cell surfaces and in their milieu. Very importantly, they not only detect the presence of molecules signaling danger via activating receptors, but can also sense the loss of self-molecules expressed constitutively under normal circumstances, the latter also termed missing-self recognition[3]. After detecting an abnormal cell, NK cells display various effector functions. They were named Natural Killer cells, because they were able to spontaneously kill tumor cells[4]. They can kill other cells by releasing cytotoxic molecules from their granules, but they are also very potent producers of cytokines and chemokines, thereby taking part in an orchestrated immune response.[5] Traditionally they were believed to only be part of the innate immune system, possessing a static receptor repertoire and a short turnover time. In recent years, however, it has been shown, that NK cell populations display an unexpected diversity and that some also possess properties of adaptive immune cells, such as long-lasting imprinting of their repertoire and memory functions.[6]

1.1.1 Adaptive NK cells

About 10 years ago, NK cells were shown to mediate a recall response in a mouse model of contact hypersensitivity with RAG-deficient mice, which lack B- and T-cells.[7] Subsequently, NK cells with adaptive properties have also been studied in other models, most extensively in infections with mouse cytomegalovirus (CMV).[8]

Adaptive NK cells have also been described in humans. Guma et al. observed an expansion of NK cells expressing the activating receptor CD94/NKG2C in individuals with a positive CMV serostatus.[9] Afterwards several adaptive features have been attributed to this subset. Those cells show a profound change in their cell surface receptor repertoire (e.g. CD2, NKG2A, KIRs) in comparison to canonical NK cells.[9,10] In addition, epigenetic changes on focal and global levels have been shown. A demethylation of the IFNG locus CNS1, which directly parallels that of memory CD8 T cells and Th1 cells, showed an imprinted link to permanent functional changes.[11] Also alterations in signaling adaptors and transcription factors characterize the NK cell expansion in individuals with positive CMV serostatus.[12] This expanded NK cell subset possesses strong effector functions in response to selected stimuli, such as triggering via NKG2C or CD16 receptors.[10,12,13] Several investigators describe the expansion of this human NK cell subset in response to CMV[14–16]. Which specific ligands or epitopes are involved in their generation, however, have only been speculated upon and probably involve redundant pathways induced by activating receptors.[10,17,18]

1.1.2 NK cell-MHC interactions and peptide specificity

Analogous to T cells, the MHC system plays an important role in interactions of NK cells, but unlike T cells, NK cells do not possess the ability to express a rearranged receptor repertoire making use of recombination activated genes (RAG). Instead they have two RAG-independent systems of receptors which detect MHC molecules. One is represented by the highly diverse family of killer-cell immunoglobulin-like receptors (KIR), including activating and inhibitory members, whose main ligands are the classical MHC class I molecules HLA-B and HLA-C.[19] The other system comprises more conserved lectin-like receptors, namely CD94/NKG2A and its activating counterpart CD94/NKG2C, which recognize the poorly polymorphic non-classical MHC class I molecule HLA-E.[20] The inhibitory and activating functions of these receptors are carried mainly by different intracellular signaling domains, while the extracellular domains responsible for ligand binding are very similar.

The inhibitory receptors recognizing their MHC ligands are important for a mechanism termed NK cell “licensing” or “education”.[21,22] NK cells expressing MHC receptors which recognize self-ligands are rendered functionally active by this mechanism. Self-specific NK cells are tolerant under steady state but are highly competent in detecting and killing target cells which have downregulated surface MHC class I molecules upon infection or malignant transformation.[3]

Also, for the activating receptors, diverse functions have been shown[23]. Different activating receptors (e.g. NKG2D, NKp30) have been shown to play a role in infection and tumor immunity.[24–26] Owing to the constitutive engagement of inhibitory receptors, the orchestration by costimulation of several activating receptors is thought to play an important role in NK cell activation.[27]

All cells containing a nucleus can express MHC I molecules. MHC class I molecules are loaded with short peptides derived from the cytoplasm (e.g. of a virus-infected cell) and these viral peptide-MHC class I complexes are recognized by the T cell receptor (TCR) expressed by CD8+ T cells, thereby initiating an immune response.

The role of peptides for NK cell-mediated recognition of MHC is now well recognized. It has been reported that the loading of different peptides on the MHC molecule can finely tune the activation and inhibition of some receptors[28–30] and that NK cells also could detect virus-encoded peptides.[31–33] Although the role of peptides in functionally shaping NK cell responses remains unclear, these findings provide another parallel to T cells and suggest that peptide-mediated recognition could be relevant for NK cell activation.

1.2 Human cytomegalovirus

Human CMV, also called Human Herpesvirus 5, is an enveloped double-stranded DNA virus which is a member of the of the Herpesviridae family. The name cytomegalovirus is derived from the enlargement of infected cells by viral inclusion bodies. Influenced by regional differences and age, the frequency of a positive CMV serostatus ranges from 60% to 100% of all individuals.[34] A primary infection is still a very serious threat in prenatal condition, after birth or in immunodeficient individuals, but will otherwise only cause a mild, unspecific clinical phenotype. Like other herpesviruses, CMV has the ability to persist permanently inside its host in a latent state after primary infection. It will then only cause a pathogenic reactivation if the control by the host´s immune system is lost. Lots of different acquired and inherited immune defects can cause such reactivation, for example infection with HIV or immunosuppression after organ transplantation. During infection/reactivation nearly all organs can be affected. Typical complications are caused by CMV hepatitis, pneumonitis, colitis and retinitis.

1.2.1 Immune evasion of CMV

A big part of the CMV genome encodes for proteins important for immunoevasion, which could explain its ability to survive latently inside the host.[34] As mentioned above, the recognition of peptide-MHC class I complexes is an important pathway of CD8+ T cell activation and CMV has to evade the MHC class I system.

CMV strains encode for many genes whose main function is related to the downregulation of HLA class I molecules by inhibiting different steps of HLA class I and peptide processing.[35–38] One important immune evading mechanism by CMV is the inhibition of transporter associated with antigen presentation (TAP) dependent peptide loading by CMV US6.[36]

On the other hand, NK cells can detect the downregulation of HLA class I, thus rendering these cells specialized for the defense against CMV.[21,22] To avoid NK cell-mediated surveillance, CMV has evolved mechanisms to retain the expression of certain host HLA class I molecules[39,40] or even codes for MHC class I-like molecules to mimic their functions as ligands for NK cell inhibitory receptors, as in the case of CMV glycoprotein UL18, which binds to LIR-1/ILT2.[41]

CMV has also evolved strategies to evade immune recognition by the inhibitory NK cell receptor CD94/NKG2A. As mentioned previously, CD94/NKG2A binds the non-classical HLA class I molecule HLA-E, which is stabilized under steady state by signal peptides derived from other HLA class I molecules[42], enabling NK cells to monitor the global HLA class I expression by NK cells, independent of the alleles expressed.[20,43,44] Interestingly, the UL40 gene of CMV contains a sequence which is homologous to those encoding for the host signal peptides of HLA class I molecules.[45] Those viral peptides are able to stabilize HLA-E and to inhibit NK cells expressing the inhibitory receptor CD94/NKG2A, thus serving as an additional immunoevasion strategy.[33,45,46] On the other hand, a contribution to CMV control by NKG2C+ NK cells has been supported by a case report.[47] It should also be mentioned that the homozygous deletions of the NKG2C locus is not such a rare event (~4% of all individuals) and that those individuals also have expansions of NK cells with phenotypic changes similar to those of individuals without NKG2C deletion.[10] This could indicate a redundant role of CD94/NKG2C in the control of CMV by NK cells.

1.2.2 CMV UL40 polymorphism

Garrigue et al. and more recently Heatley et al. have shown that the CMV UL40 locus encoding for the signal peptide which stabilizes HLA-E is highly polymorphic.[33,48,49] The latter group further tested those peptides for their ability to stabilize HLA-E in transfected cell lines and their ability to interact with both CD94/NKG2A and CD94/NKG2C by surface plasmon resonance analysis and observed profound differences between the peptides. The fact that some CMV strains possess sequences encoding for signal peptides which are non-functional suggests a selective pressure by the activating receptor CD94/NKG2C in CMV infections. Taken together this means that different CMV strains could have developed different strategies to adapt to particular conditions within the host. This would support a model in which, over a long period of time, coevolution led to a stalemate situation between CMV and the human immune system

1.3 Allogeneic Hematopoietic stem cell transplantation (alloHSCT)

The transplantation of hematopoietic stem cells was first successfully carried out in the late 50s with bone marrow transplantation in syngeneic twins.[50] With the discovery of the HLA system stem cell transplantation later became also possible from matched unrelated donors. Over the recent decades, the indications as well as patients who could be treated with this cell-based therapy gradually increased. The most common indications for an alloHSCT are nowadays hematologic malignancies, other hematologic disorders and inherited genetic defects involving the hematopoietic system. After initially being used mainly as a kind of supportive therapy to enable immune reconstitution after myeloablative radio-chemotherapy in leukemia, the importance of the graft itself in the treatment of malignancies is being emphasized nowadays.

The discovery of the importance of the graft-vs-leukemia effect was the basis for the introduction of reduced-intensity conditioning, which made this therapy also available for patients with advanced age and morbidity[51]; it is now used more often than full

myeloablation. Patients undergoing alloHSCT still have a high mortality, caused by infections, graft-versus-host disease and relapse of the underlying disease. That is why supportive therapy (e.g. prophylactic immunosuppressive treatment of GvHD and graft rejection as well as preemptive/prophylactic treatment of infections) plays such a central role in patients undergoing stem cell transplantation.

1.3.1 AlloHSCT and CMV

During alloHSCT, the impaired immune response induced by conditioning as well as the treatment with immunosuppressive drugs facilitate CMV reactivation from its latent state and can cause a symptomatic infection of different organs. A preemptive strategy with regular monitoring of CMV-DNAemia by PCR and immediate treatment before clinical overt disease has reduced morbidity and mortality caused by CMV[52] and is now the standard after alloHSCT. The highest risk factor for CMV DNAemia is a positive CMV serostatus of the recipient.[53] About 50 to 60% of all CMV seropositive recipients will have detectable levels of CMV DNA after alloHSCT. Most reactivations happen within the first two months after transplantation, but they can even occur years after transplantation due to immunosuppressive treatment.[54]

Even though the clinical impact of CMV in alloHSCT remains controversial, there is some data reporting a lower relapse rate in patients with early CMV reactivation, suggesting that CMV can promote an anti-leukemia effect.[55,56] However, the overall survival rate, because of an increase in non-relapse mortality, remained unchanged.

1.3.2 NK cells after alloHSCT

NK cells are the first lymphocytes to recover after alloHSCT and might exert a beneficial effect as supported by data showing that NK cells can display both anti-tumor as well as anti-viral responses, while not causing or even protecting from GvHD.[57,58] Indeed, several studies have linked different NK cell responses to an improved clinical outcome.

Patients with an early NK cell reconstitution have an improved survival rate.[59] Moreover, patients receiving a graft with NK cells having an HLA-matched KIR and thereby having the ability of NK cell functional maturation by licensing show a strongly improved survival.[60,61] Last but not least, several studies investigating cell-based therapies with NK cell infusions have also shown some promising results.[62–65]

The important role of NK cells after alloHSCT and the profound change of certain NK cell populations in CMV seropositive healthy individuals led to studies which showed that NK cells are also shaped by CMV reactivation after alloHSCT.[17,66] Like CMV seropositive individuals, also patients with CMV reactivation showed an expansion of NK cells expressing the activating receptor CD94/NKG2C, an NK cell subset displaying higher functional capacities. Two recent studies tried to link the expansion of this NK cell subset to a clinical outcome.[67,68] The results, however, were contradictory. While one group observed a reduced relapse rate in patients with CMV reactivation attributed to an increase of expanded NK cells, the other group observed an increased relapse rate in patients with NK cells displaying an adaptive phenotype. The authors of the second study discuss the differences found and attributed them to a different source of stem cells (peripheral HSCT against umbilical cord stem cell transplantation), as well as a longer follow-up during their study (with mostly protection from early relapse found in the first study). Also the time of the assessment of the NK cell phenotype is different between the two studies, with 6 months after transplantation in the first study and 9 to 12 months after transplantation in the second study. One should note that due to early mortality a substantial part of patients also had already died by then and thus were not included in the analysis of the two studies.

1.4 Hypothesis

Several receptor affinity studies have shown that different signal peptides bound to the non-classical MHC class I molecule HLA-E strongly influence the binding to the receptors CD94/NKG2A and CD94/NKG2C.[20,33] As previously outlined, the gene locus in CMV which carries the sequence for this signal peptide seems to be highly polymorphic.[33]

It remains unknown whether the influence on the receptor-ligand interaction between HLA-E and the activating receptor CD94/NKG2C has an impact on functionality or even the generation of adaptive NK cells. If this should be the case, then different CMV strains would shape the CD94/NKG2C expansion of NK cells differently.

To show a relevance of this interaction in humans, patients undergoing alloHSCT serve as a good model. First of all, these patients have a high incidence of CMV reactivation, which is necessary to sequence the CMV UL40 gene. Secondly, NK cells play an important role in the clinical outcome after alloHSCT. Different interaction potentials of CMV encoded peptides could then influence the clinical outcome.

Based on these premises, I formulated the hypothesis that different CMV UL40 encoded peptides impact on shaping the repertoire and the functionality of adaptive NK cells, thereby potentially influencing the clinical outcome and viral control after alloHSCT.

In order to test this hypothesis in my thesis, I have investigated the following points:

1. To analyze CMV UL40 signal peptide sequence polymorphism in our own cohort
2. To classify different CMV UL40-derived peptides by functional impact on NK cells
3. To investigate the impact of different CMV strains classified by functionality of CMV UL40 sequence on the shaping of adaptive NK cells in the patients
4. To analyze the influence of adaptive NK cells on the clinical outcome and viral control after alloHSCT

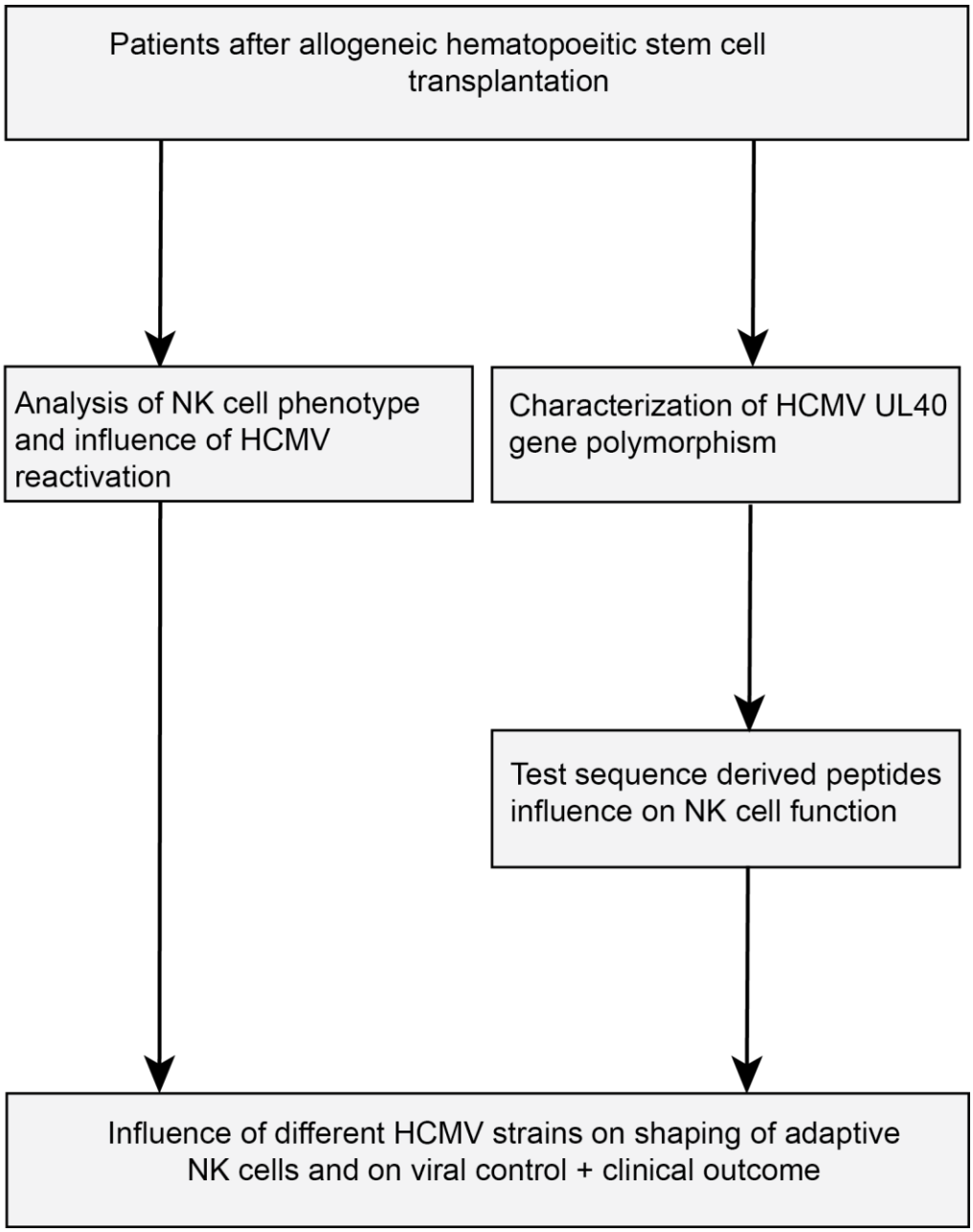


Figure 1: Workflow diagram

2 Patients and Methods

2.1 Patients

All experimental procedures were performed with permission from the local ethics review board in Berlin, Germany. Patients and blood donors who participated in this study gave written informed consent (Charite ethics committee approval EA1/1/169/14). Buffy coats from healthy blood donors were obtained from “Deutsches Rotes Kreuz Blutspendedienst Nord-Ost” in Dresden (EA1/149/12). All patients undergoing alloHSCT at the Hematology Department of the Charité Hospital between November 2014 and December 2015 were asked to participate in this study. 40ml of blood was drawn weekly after alloHSCT while on the transplantation ward and thereafter selected patients were monitored at outpatient clinics. The plasma samples used for the qPCR monitoring of CMV copy numbers were obtained from the department of laboratory diagnostics (Labor Berlin GmbH) and used for CMV PCR and sequencing. Patient characteristics are summarized in **Table 1**.

Patients treated with a myeloablative regimen received cyclophosphamide on two consecutive days (Cy; 60 mg/kg/d) and 6 x 2-Gy total body irradiation (TBI). In most cases patients with non-myeloablative conditioning were treated with fludarabin (6 x 30 mg/m²) and busulfan (2 x 4 mg/kg), but also different regimens were used. All patients were treated with antithymocyte globulin (ATG) before alloHSCT, either with 3x10 mg/kg or with 4x10 mg/kg. Prophylaxis of aGvHD (acute Graft versus host disease) contained cyclosporine A (CSA) adjusted to blood levels and either methotrexate (MTX; 15 mg/m² at day 1 and 10mg/m² at day 3, 6 and 11) or mycophenolate mofetil (MMF; 4 x 500mg).

The patients were monitored weekly for CMV after alloHSCT and less stringently at later stages in the outpatient clinic. The CMV copy number was monitored by qPCR and treatment was initiated if it exceeded 2000 copies/ ml. Patients were most commonly treated with oral valganciclovir, but intravenous ganciclovir or foscarnet was also used.

Variable	**CMV reactivation**	**no reactivation**	**p**
n	47	42	
Age			0.14
median	57	60,5	
(range)	(21-74)	(29-75)	
Gender			0.83
Female	19	18	
Male	28	24	
CMV serostatus			<0.01
R+D+	29	8	
R+D-	13	7	
R-D+	3	8	
R-D-	0	19	
unknown	2	0	
Diagnosis			0.95
AML	37	32	
CR1	20	17	
CR2	8	6	
persistence	9	9	
ALL	2	2	
MDS	3	2	
other	5	6	
NHL	2	0	
CML	0	2	
CLL	1	0	
CMML	1	1	
OMF	1	2	
MM	0	1	
Graft status			0.78
10/10	39	36	
sibling	2	5	
09/10	8	6	
Conditioning			0.78
RIC	39	36	
MA	8	6	
GvHD prophylaxis			0.32
CsA + MMF	37	29	
CsA + MTX	9	12	
unkown	1	1	
Graft vs Host disease			0.45
aGvHD≥°2	7	11	
Clinical outcome			0.20
died or relapse	22	14	
relapse	13	12	
infectious	7	1	
aGvHD	2	1	
previous HSCT			0.42
n	2	4	

Table 1: patient characteristics

2.2 Methods

2.2.1 Nested PCR and Sanger sequencing

DNA from plasma during CMV reactivation was isolated using the QIAamp® DNA micro kit (Qiagen) in accordance with the manufacturer's instructions. For the nested PCR, the following primers were used:

Outer PCR forward: 5'-GGCTCTGTCTCGTCGTCATT-3'

Outer PCR reverse: 5'-TAAGGGCACTCGTGAGGATG-3'

Outer PCR product : 792 base pair length

Inner PCR forward: 5'-CAACAGTCGGCAGAATGAAC-3'

Inner PCR reverse: 5'-CTGGAACACGAGCGGACATA-3'

Inner PCR product: 200 base pair length

Table 2 shows the reaction mix and cycler settings used for the nested PCR and **Figure 2** shows an exemplary picture of a gel after electrophoresis of PCR products and Gene Ruler 1kb plus ladder (Thermo Fisher Scientific).

We had to adjust the settings of the nested PCR during the experiments because of contamination and subsequent amplification with an overcycled PCR product, producing a smear in the gel electrophoresis. At first, we used Platinum® *Taq* DNA Polymerase, Primer annealing temperature of 58°C for inner and outer PCR as well as 35 cycles instead of 30 for the inner and outer PCR.

For most of the PCR reaction, we then used AccuPrime™ *Taq* DNA Polymerase, high fidelity (ThermoFisher scientific) and the settings in **Table 2**.

The product of the nested PCR was sent to commercially available Sanger sequencing with the reverse primer of the inner PCR (Mix2Seq by Eurofins genomics).

Amplified PCR products from some patients repeatedly showed bad quality in Sanger sequencing, which we were able to attribute to a polymorphism at the end of the primer binding site (**Figure 3**). The quality in those samples could be improved by the use of a shorter reverse primer not containing the base which bound to the polymorphic site (Primer 2). This primer was used for most of the sequencing results obtained.

Outer PCR Mix

Accu Prime Buffer I	2.5 µl
UL40 Outer Primer Mix 10µM each	1 µL
Accu Prime Polymerase	0.1 µL
H_2O	11.4 µl
Isolated DNA	10 µl

Cycler settings

Initial Denaturation	94°C 90 seconds	
Denaturation	94 °C 30 seconds	
Primer Annealing	57 °C 30 seconds	**30x**
Primer Elongation	68 °C 60 seconds	
Final elongation	68 °C 600 seconds	
Pause	4 °C	

Inner PCR Mix

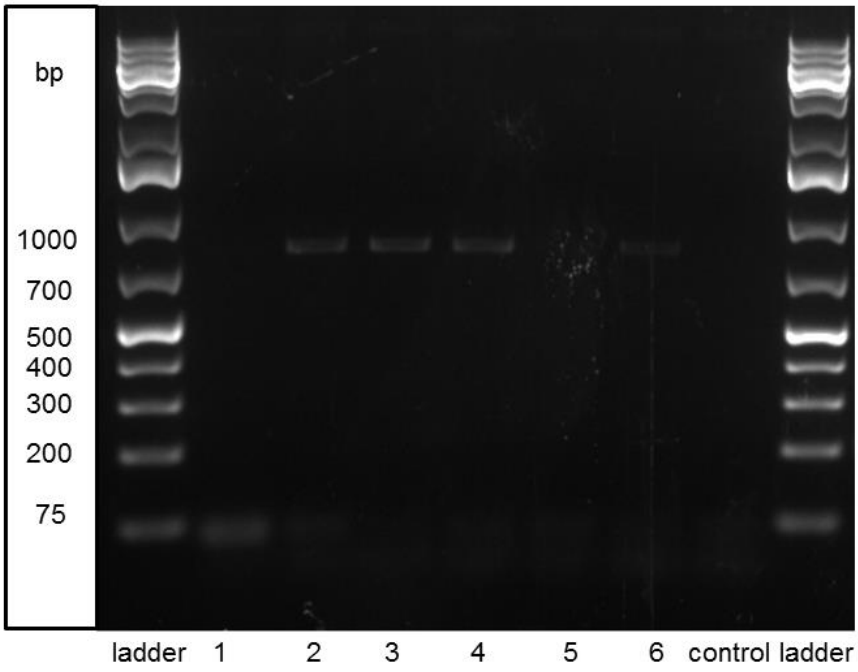
Accu Prime Buffer I	2.5 µl
UL40 Inner Primer Mix 10µM each	1 µL
Accu Prime Polymerase	0.1 µL
PCR product of outer PCR	1 µl
H_2O	20.4 µl

Cycler settings

Initial Denaturation	94°C 90 seconds	
Denaturation	94 °C 30 seconds	
Primer Annealing	57 °C 30 seconds	**30x**
Primer Elongation	72 °C 30 seconds	
Final elongation	72 °C 600 seconds	
Pause	4 °C	

Table 2: nested PCR properties

Outer PCR



Inner PCR

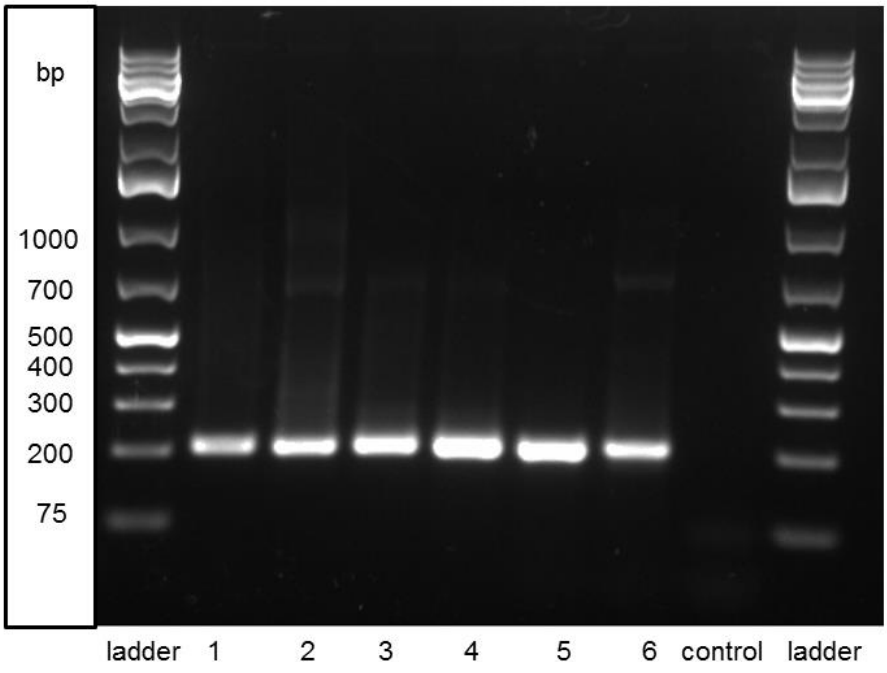


Figure 2: gel electrophoresis of PCR products from nested PCR with non-template control

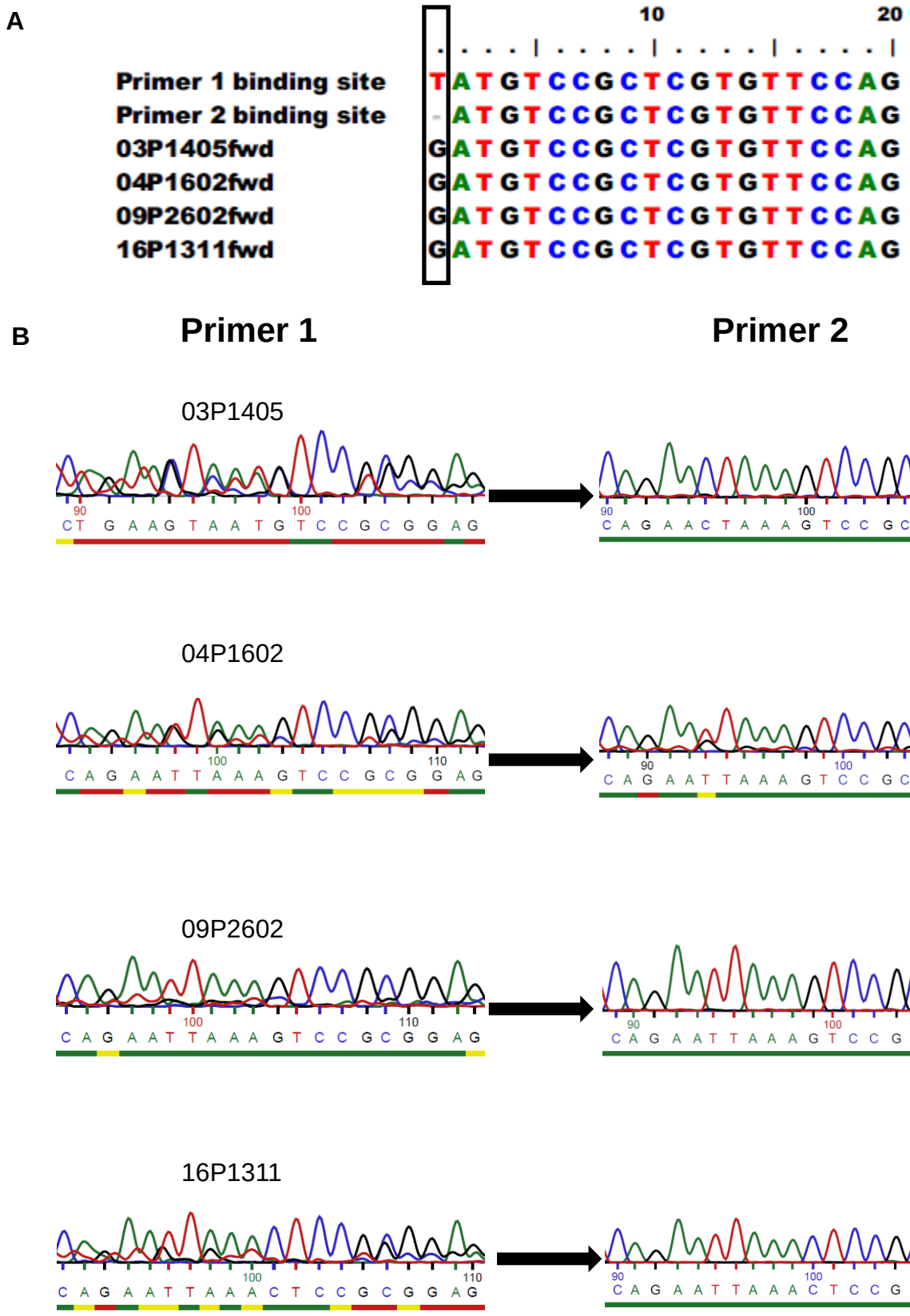


Figure 3: Low quality sequencing caused by polymorphism at the primer binding site. (**A**) Nucleotide sequences of primer binding site from the two different primers used and sequences of primer binding site from 4 patients with low quality sequencing results. (**B**) Sequencing raw data of 4 patients with low quality sequencing results and improved quality with new primer

2.2.2 Cloning of PCR products

Inner PCR products for cloning were purified by the use of the QIAquick® PCR purification Kit (Quiagen) in accordance with the manufacturer's instructions. Purified PCR products were poly-A tailed with the following reaction mix by incubation for 30 minutes at 70°C:

4.4µl purified PCR product, 2µl 5xgoTaq buffer (Promega), 0.6 µl 25mM MgCl2 (Promega), 4µl dATP (0.5m; NewEngland Biolabs), 1µl goTaq polymerase (Promega)

The poly-A-tailed PCR products were then roughly quantified on an agarose gel with the use of Image Lab (Bio Rad) as software and the GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific) as reference. Cloning into a vector and transformation into competent E. coli (JM109) was performed with the use of the pGEM®-T Easy Vector Systems kit (Promega) according to the manufacturer's instruction with a molar ratio of PCR product to plasmid of 3:1 or 1:1.

For the transformation SOC medium (ThermoFisher Scientific) was used and transformed cells were plated on agar plates (LB-Agar capsules MP biomedicals according to instructions) containing Ampicillin (100µg/ml), X-Gal (40mg/ml) and IPTG (1M). After incubation over night at 37°C colonies with the plasmid containing the PCR product could be detected by white/blue screening. Twelve white colonies per PCR product were picked and transferred into LB-Medium (LB-Medium capsules MP biomedicals according to the manufacturer's instructions) and incubated shaking overnight at 37°C. Plasmid DNA was then purified with the use of the NucleoSpin plasmid purification kit (Machery-Nagel) according to the manufacturer's instructions. Afterwards the PCR product sequence was amplified with the inner PCR of the nested PCR (see 2.2.1) and Sanger sequencing was performed with the Mix2seq kit (send to Eurofins genomics).

2.2.3 Sample preparation, cryopreservation and NK cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples and buffy coats obtained from patients and healthy donors by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Life Sciences).

Cells were stored in liquid nitrogen in FCS supplemented with 10% DMSO or directly used for further experiments. After thawing of stored cells, they were rested in complete medium (RPMI1640 containing glutamine and supplemented with 10% FBS, 20 µM β-mercaptoethanol, and 100 U/mL Penicillin-Streptomycin; all Thermo Fisher) at 37°C overnight before further use.
For the use of NK cells in the stimulation assay, CMV seropositive donors were screened with FACS for an expanded phenotype according to Hammer et al.[69] Before freezing, CD56+ cells were enriched by Magnetic cell separation using CD56 MicroBeads (human, Miltenyi) in an AutoMACS in accordance with the manufacturer's instructions. Cells were cryopreserved in FBS containing 10% DMSO.

2.2.4 Antibodies and flow cytometry

Table 3 shows all antibodies used. All samples were stained with a Live/Dead viability dye. Intranuclear stainings were performed using the FOXP3 fix/perm buffer set (eBioscience™). For the intracellular staining of cytokines in the stimulation assay, cells were permeabilized with BD FACS™ Permeabilizing Solution 2 (BD Biosciences) The samples were acquired with a Fortessa 15 color flow cytometer (BD Biosciences) and further analysed by FlowJo Version 10.

2.2.5 Cell lines

For the experiments, two different tumor cell lines were used. The human erythroleukemia cell line K562 transfected with HLA-E (K562/E) and the mouse lymphoma cell line RMA-S transfected with HLA-E (RMA-S/E). Both cell lines were maintained in complete medium. For selection of transfectants K562/E were supplemented with the antibiotic G418 (Invivogen) at a concentration am 1mg/ml and RMA-S/E with Hygromycin B (Invivogen) at a concentration of 400µg/ml.

2.2.6 Testing of HLA-E stabilization

RMA-S/E transfectants with a cell concentration of 1x10e6/ml were pulsed with different peptides (synthesized by Peptides & Elephants GmbH) overnight at 300µM concentration in Gibco™ Opti-MEM™ I Reduced Serum Medium (ThermoFisher scientific supplemented with penicillin and streptomycin). The cells were then washed and stained with HLA-E PE-Cy7 (clone 3D12, BioLegend), viability dye APC-eFluor780 (eBioscience) and FcR block (clone 2.4G2, DRFZ).

Antigen(clone)	**dye**	**manufacturer**
viability	Zombie Aqua™	BioLegend
viability	APC-eFluor780	eBioscience
CD14(61D3)	BV510	BioLegend
CD19(HIB19)	BV510	BioLegend
CD56 (HCD56)	PE-Dazzle594	BioLegend
CD3 (UCHT1)	PE-Cy5	BioLegend
CD57 (TBO1)	Purified	eBioscience
NKG2A (REA110)	PE-Vio770	Miltenyi
NKG2A (REA110)	Biotin	Miltenyi
NKG2C (REA205)	PE	Miltenyi
CD2 (RPA-2.10)	PerCP-Cy5.5	BioLegend
LIR1α (HP-F1)	APC	eBioscience
CD7 (M-T701)	BV786	BD Biosciences
Siglec-7 (REA214)	APC-Vio770	Miltenyi
FcεRIγ (polyclonal)	FITC	Merck
Ki-67 (Ki-67)	Alexa Fluor 700	BioLegend
αIgM (RMM-1) for CD57	BV605	Biolegend
Mouse FcR block(2.4G2)	-	DRFZ (in house)
HLA-E (3D12)	PE-Cy7	BioLegend
Streptavidin	BV785	BioLegend
αCD107a (eBioH4A3)	Alexa Fluor 488	eBioscience
TNFα (Mab11)	BV605	BioLegend
IFN-γ (45-15)	PE-Vio770	Miltenyi
CCL3 (REA257)	APC	Miltenyi

Table 3: antibodies used for flow cytometry

2.2.7 Stimulation of PBMCs from healthy donors

K562/E transfectants were pulsed with different peptides as described for RMA-S/E. Frozen CD56+ cells from CMV seropositive donors with an NK cell expanded phenotype were thawed as previously described, sorted for viable CD3- CD56+ NK cells by the cell sorter staff at the DRFZ at a FACS BD ARIA or ARIAII and afterwards rested overnight in complete medium. Peptide pulsed K562/E and rested NK cells were washed once and then co-cultured at an effector:target ratio of 2:1 in serum-free Opti-MEM™ medium with peptides supplemented at a concentration of 300µM. For the assessment of degranulation, αCD107a (eBioH4A3, eBioscience) was added before starting the stimulation. The stimulation was started by centrifugation at 30g for 3 minutes. After one hour GolgiPlug™ and GolgiStop™ (both BD biosciences, containing Brefeldin A and Monensin) were added for the assessment of intracellular cytokines. Stimulation was stopped at 6 hours total time. Cells were washed once, stained for cell surface markers and intracellular cytokines before analysis and fixated with paraformaldehyde 2% before analysis with a FACS BD Fortessa.

2.2.8 Statistical Analysis

Statistical analysis was performed with Graph Pad Prism® Version 5. Categorical data analysis was performed by Fisher´s test for 2 variables and the Chi square test for comparison of more than 2 variables. For comparison of continuous data (FACS data) between two groups, the Mann-Whitney U test was used. Regression analysis was also performed with Graph Pad Prism® Version 5.

3 Results

3.1 Intra- and interhost variability of CMV UL40 sequences encoding for leader peptides

Previous analysis of the UL40 sequence encoding for HLA-E stabilizing peptides derived from a limited number of HCMV strains has already indicated that this sequence is highly polymorphic in patients with CMV reactivation[33]. To assess the influence of CMV strains encoding for different leader peptides on NK cell phenotype and functionality, we first had to sequence CMV UL40 in our own cohort of patients with CMV reactivation, which we succeeded in 34 patients. (**Figure 4**) Interestingly, the analysis of the Sanger sequence data showed double peaks inside the sequence encoding for the leader peptides in 7 of 34 patients with CMV reactivation, which is not surprising, as intrahost variability of CMV has already been described.[48,49] By cloning PCR products into a vector, bacterial transformation and sequencing of single colonies, we were able to show that those double peaks were caused by different PCR products likely reflecting different CMV strains inside the host (Figure 1c). Inside the part of the CMV UL40 gene we sequenced, the sequence encoding for the leader peptide was much less conserved compared to the neighboring part of the sequence (Figure1a). Even though there were two other dimorphisms near the leader peptide sequence (V to L and C to S), polymorphisms with stronger changes of the amino acid properties (for example I to F at position 8 of the leader peptide) were only found inside the leader peptide sequence.

Inside the leader peptide sequences, polymorphisms were found at all positions except position 7 (Figure 1b). The amino acid at Position 8, which is known to crucially influence the affinity of the HLA-E/peptide to NKG2/CD94 receptors[70], showed the weakest conservation of all positions.

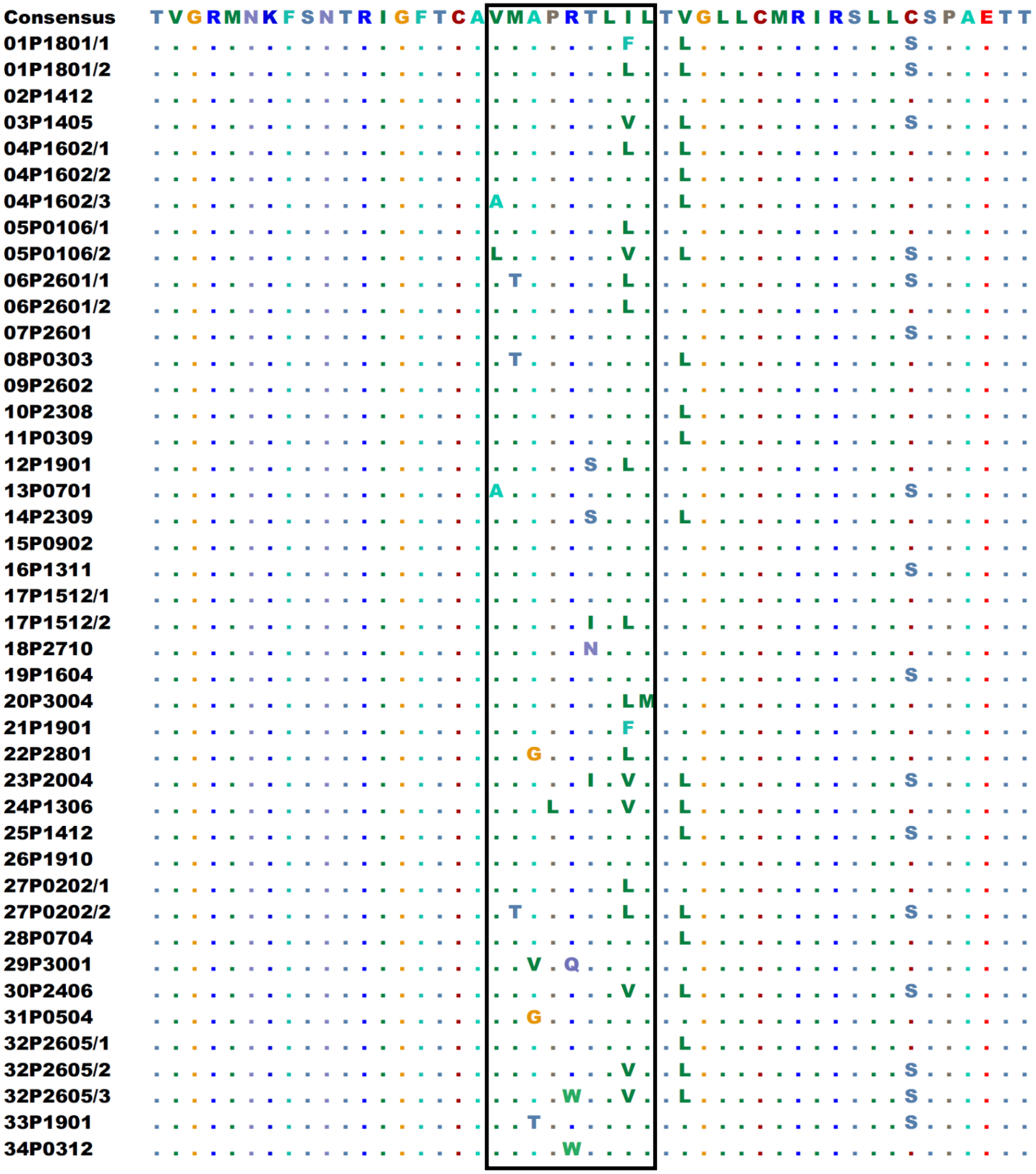
The cloning of the PCR products and sequencing of single transformed colonies of competent bacteria was also used to determine the quality of the sequencing data.

Of 132 clones sequenced, which were derived from 12 different patient samples, only one clone showed a sequence not previously detected as peak in sanger sequencing of the not-cloned PCR product. Taken together with the fact that every PCR and sequencing been performed at least twice, this speaks for a high reproducibility of our sequencing data.

3.2 Nonameric peptides derived from CMV UL40 sequences present in patient cohort differ in their ability to stabilize HLA-E

The interaction between HLA-E-bound peptides and NKG2C should be influenced not only by the affinity, but also by the expression level of the ligand. That is why we tested the ability of the different nonameric peptides derived from polymorphic CMV UL40 sequences to stabilize HLA-E. **Figure 5** shows the mean fluorescence intensity of an HLA-E-transfected cell line pulsed with the different nonameric peptides at saturating concentrations. All peptides were able to increase the surface level of HLA-E compared to cells not pulsed with any peptide. The surface levels between the different peptides were, however, very different. Cells pulsed with the peptide VMGPRTLIL showed the lowest expression level of HLA-E. In their study Heatley et al.[33] tested the ability of different peptides to stabilize HLA-E. The peptide VMGPRTLIL also induced the lowest expression level of HLA-E in their study, which is in line with our results.

A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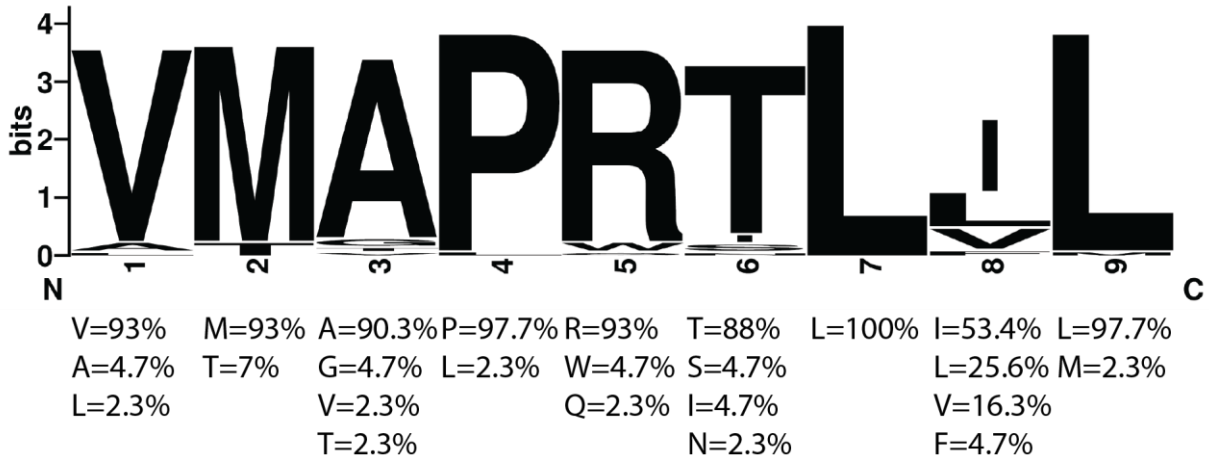
01P1801/2 . . . . . L . . L . . . . . S . . . . . .
02P1412 . . . . . .
03P1405 . . . . . V . . L . . . . . S . . . . . .
04P1602/1 . . . . . L . . L . . . . . .
04P1602/2 . . . . . L . . . . . .
04P1602/3 . . . . . A . . . . . L . . . . . .
05P0106/1 . . . . . L . . . . . .
05P0106/2 . . . . . L . . . . . V . . L . . . . . S . . . . . .
06P2601/1 . . . . . T . . . . . L . . . . . S . . . . . .
06P2601/2 . . . . . L . . . . . .
07P2601 . . . . . S . . . . . .
08P0303 . . . . . T . . . . . L . . . . . .
09P2602 . . . . . .
10P2308 . . . . . L . . . . . .
11P0309 . . . . . L . . . . . .
12P1901 . . . . . S . L . . . . . .
13P0701 . . . . . A . . . . . S . . . . . .
14P2309 . . . . . S . . . L . . . . . .
15P0902 . . . . . .
16P1311 . . . . . S . . . . . .
17P1512/1 . . . . . .
17P1512/2 . . . . . I . L . . . . . .
18P2710 . . . . . N . . . . . .
19P1604 . . . . . S . . . . . .
20P3004 . . . . . L M . . . . . .
21P1901 . . . . . F . . . . . .
22P2801 . . . . . G . . . L . . . . . .
23P2004 . . . . . I . V . . L . . . . . S . . . . . .
24P1306 . . . . . L . . . V . . L . . . . . .
25P1412 . . . . . L . . . . . S . . . . . .
26P1910 . . . . . .
27P0202/1 . . . . . L . . . . . .
27P0202/2 . . . . . T . . . . . L . . L . . . . . S . . . . . .
28P0704 . . . . . L . . . . . .
29P3001 . . . . . V . Q . . . . . .
30P2406 . . . . . V . . L . . . . . S . . . . . .
31P0504 . . . . . G . . . . . .
32P2605/1 . . . . . L . . . . . .
32P2605/2 . . . . . V . . L . . . . . S . . . . . .
32P2605/3 . . . . . W . . V . . L . . . . . S . . . . . .
33P1901 . . . . . T . . . . . S . . . . . .
34P0312 . . . . . W . . . . . .

B



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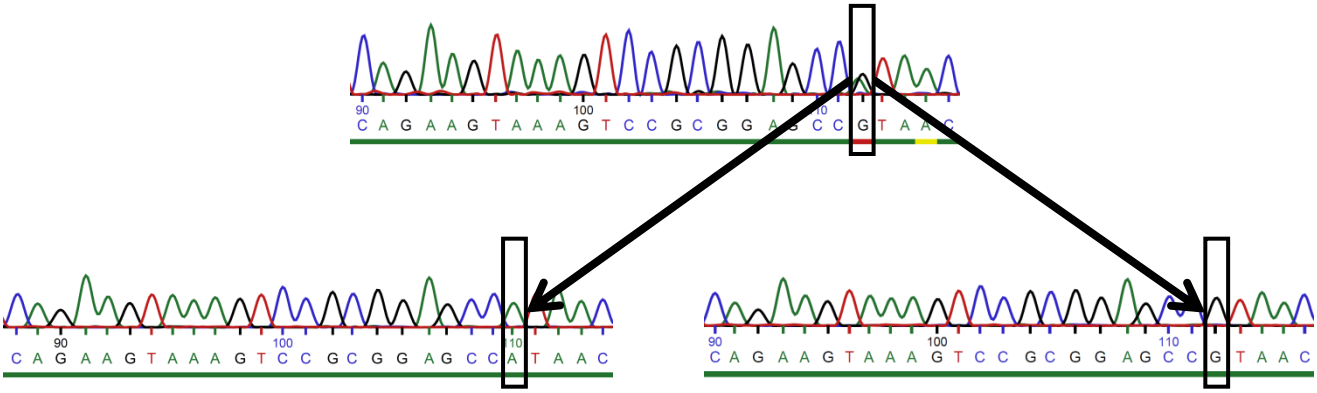
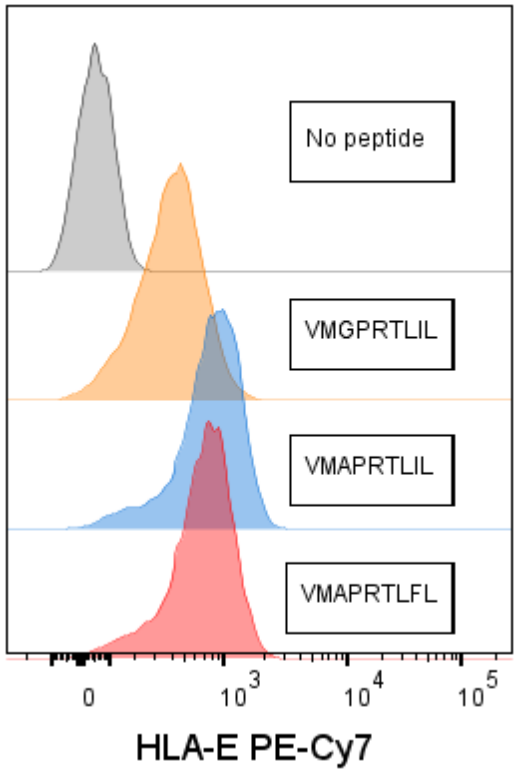


Figure 4: CMV UL40 encoding sequence shows high intra- and interhost variability. (**A)** Translated sequences from 34 patients with CMV reactivation, sequence of nonameric leader peptide is marked with frame (**B**) Sequence logo displaying the conservation of the nonameric peptide (**C**) Sanger sequencing raw data from one patient with CMV intrahost variability and results of single-colony sequencing after cloning of the PCR product into a vector

A



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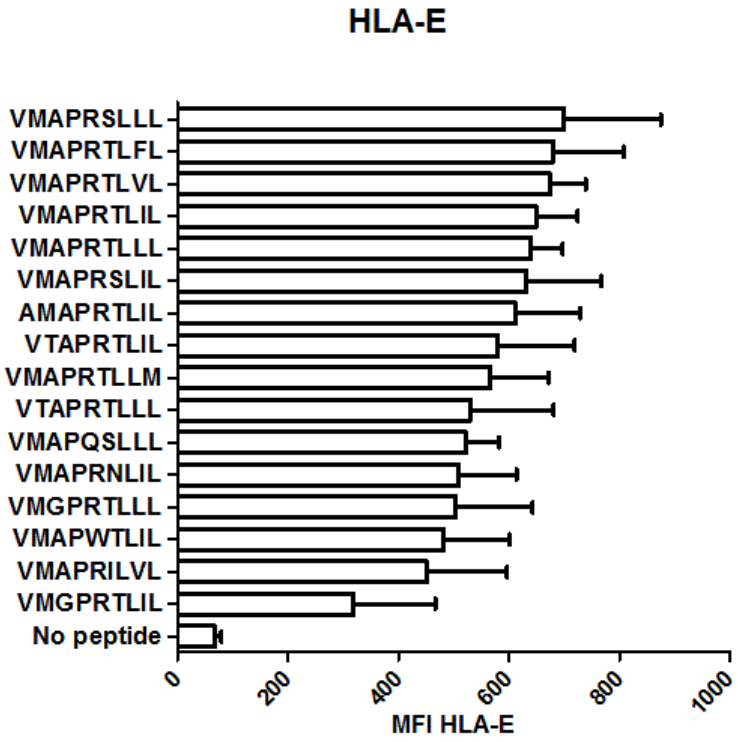


Figure 5: CMV UL40-derived peptides differ in their ability to stabilize HLA-E. (**A**) Representative FACS data showing the expression of HLA-E on RMA-S/E cells after incubation with different peptides and (**B**) mean MFI and standard deviation with four experiments

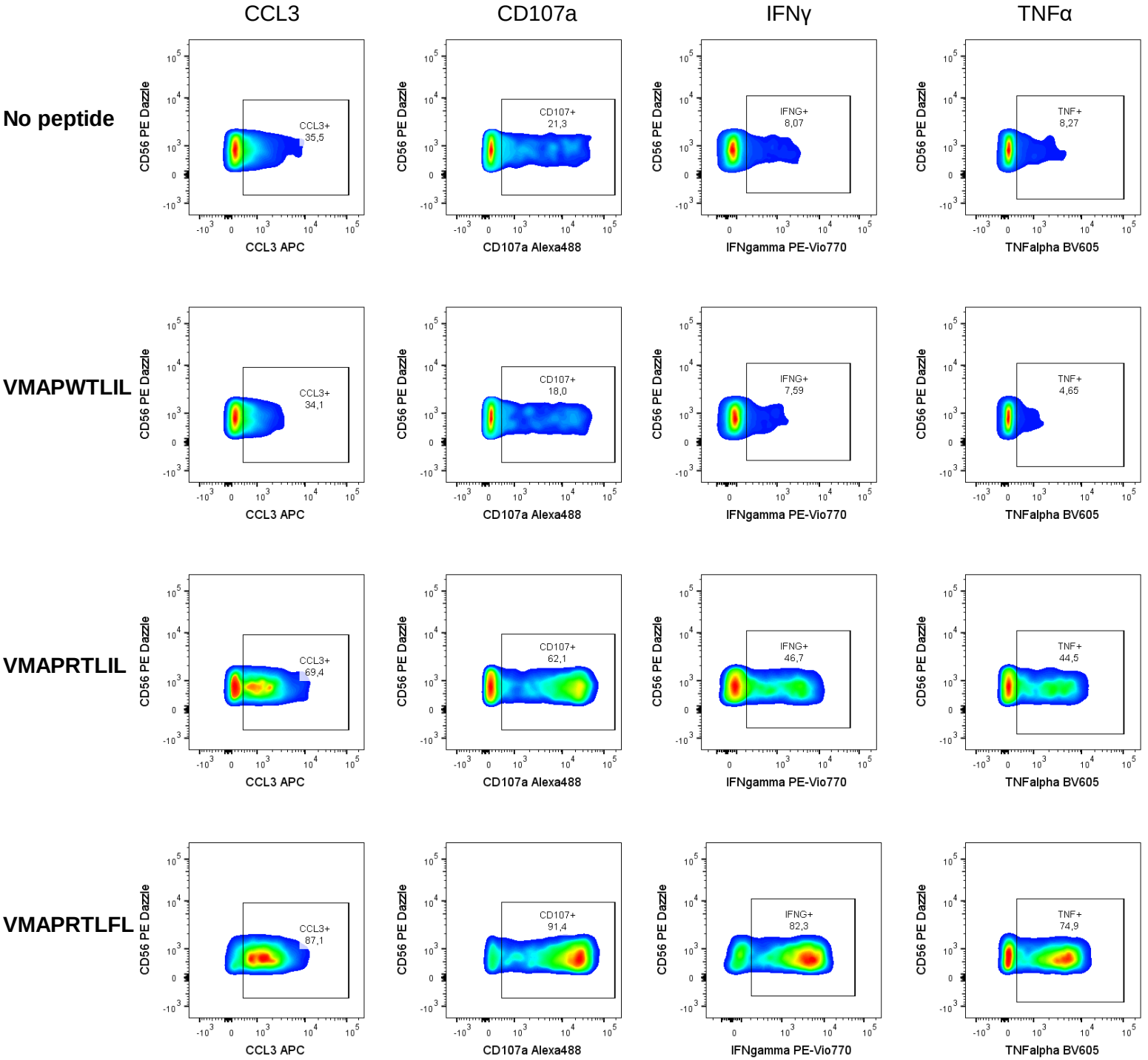
3.3 UL40-derived peptides differently activate NK cells and NKG2A+ NK cells respond more sensitively than NKG2C+ NK cells

In order to compare the NK cell properties of patients infected with different CMV strains, grouping of the different peptides according to the functional properties seemed necessary, because many different peptides (we detected CMV strains encoding for 21 different peptides in 34 patients) would have made a further comparison of the NK cell properties between the patients rather difficult. To do so we used an NK cell stimulation assay. NK cells from CMV-seropositive healthy donors with an expanded phenotype were stimulated by an HLA-E-expressing cell line (human erythroleukemia cell line K562 transfected to express HLA-E) which had been pulsed by the different nonameric peptides. It is well-know that K562 without transfection of HLA-E already causes a cytotoxic response of NK cells.[71] This baseline stimulation of NK cells makes it possible to measure the inhibition of NKG2A+ NK-cells (decrease of the baseline effector functions) and the activation of NKG2C+ NK-cells (further increase of the baseline effector functions) by peptide-pulsed HLA-E-transfected K562 cells at the same time. By determining the response by FACS-measurement of different effector functions (**Figure 6A+B**) we could see peptide dependent differences in the activation of NKG2C+ NK cells and the inhibition of NKG2A+ NK cells (**Figure 6C+D**). As expected, the activation of NKG2C+ NK cells and inhibition of NKG2A+ NK cells negatively correlate with each other (**Figure 6E**) and one can also observe the different threshold of the activation markers (CCL3<CD107a<TNFalpha≤IFNgamma), which was previously defined by other investigators.[72] It is known that NKG2A has a higher affinity to the HLA-E/peptide complex than NKG2C[73] and accordingly, our data suggests a higher sensitivity of NKG2A to peptide changes than NKG2C. For example, NKG2A+ NK cells stimulated with VMAPRTLIL were already fully inhibited showing no effector functions, which was the case for stimulation with VMAPRTLFL as well. NKG2C+ NK cells could still respond to subtle peptide changes (e.g. much stronger effector functions with VMAPRTLFL than with VMAPRTLIL), making those cells more peptide-specific, at least in our model with saturated peptide doses. The peptide which displayed the strongest activation of NKG2C+ NK cells, VMAPRTLFL, is already known to have the strongest affinity to this receptor compared to other peptides.[73,74]

Peptides with relevant amino acid changes at position 5 (R to W or Q) or 6 (T to N or I), which are known to be important for the interaction of the peptide with the CD94/NKG2 receptors[75], showed the lowest activation and inhibition of NKG2C+ and NKG2A+ NK cells. The peptide VMGPRTLIL, which showed the weakest stabilization of HLA-E, also showed a comparably low potential to activate and inhibit NK cells. As previously outlined, I wanted to simplify the data and group the different peptides in order to use this data for the analysis of the influence of different CMV strains on the NK cells in patients undergoing alloHSCT. Based on our data, the peptide sequences and previous affinity studies available[33], I classified the peptides into three categories (**Table 4**).

Of course, those groups are not completely homogeneous, e.g. some of the peptides inside the intermediately interacting group also showed a slightly lower activation and inhibition potential. Also, the grouping is influenced more by the differences in the activation of NKG2C+ NK cells, as there was no difference for VMAPRTLFL in NKG2A+ NK cells from the “intermediately interacting” peptides.

A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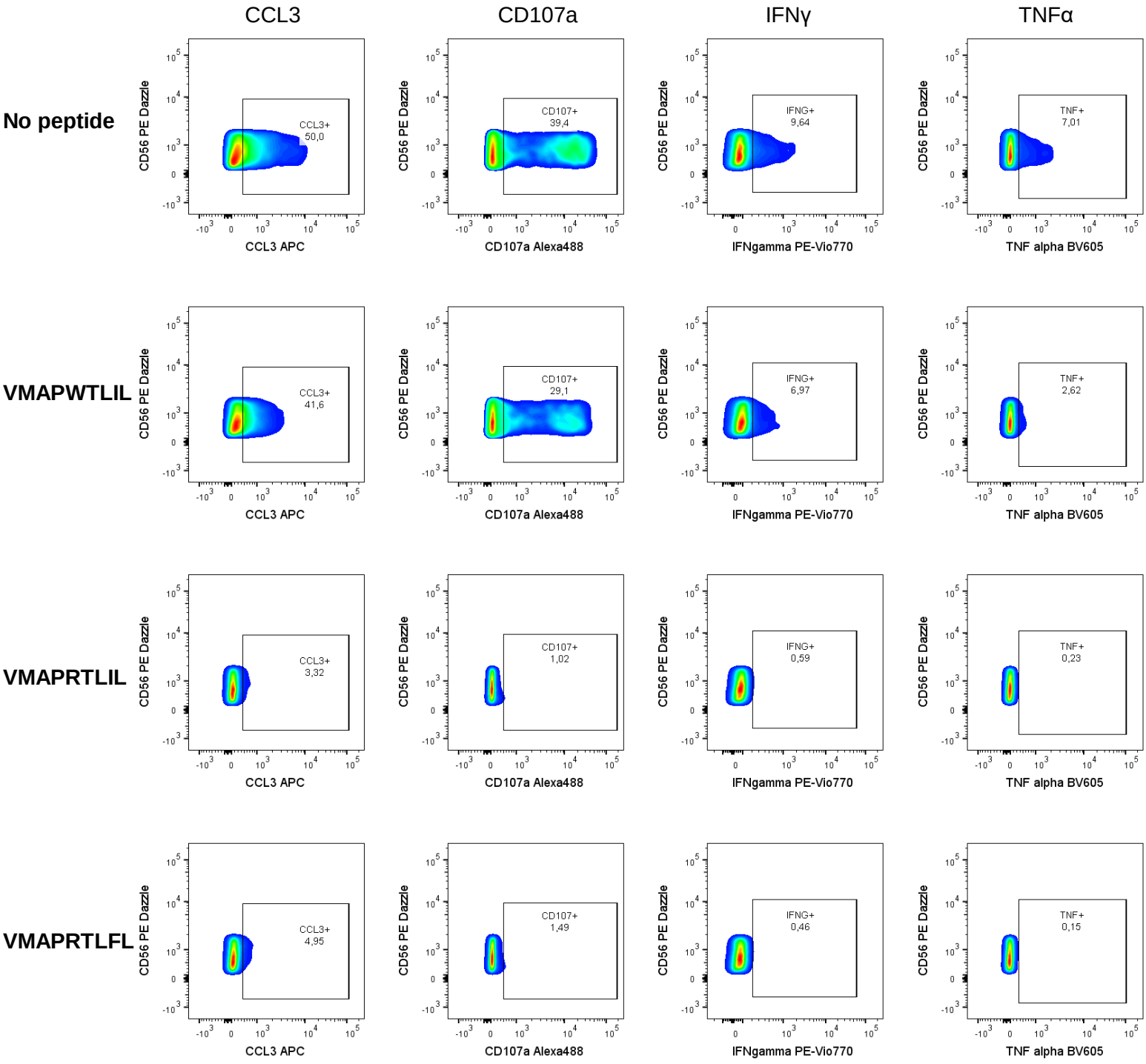
CD107a
IFNγ
TNFα
No peptide
VMAPWTLIL
VMAPRTLIL
VMAPRTLFL
CD56 PE Dazzle
CCL3 APC
CD107a Alexa488
IFNgamma PE-Vio770
TNFalpha BV605
CCL3+ 35,5
CD107+ 21,3
IFNG+ 8,07
TNF+ 8,27
CCL3+ 34,1
CD107+ 18,0
IFNG+ 7,59
TNF+ 4,65
CCL3+ 69,4
CD107+ 62,1
IFNG+ 46,7
TNF+ 44,5
CCL3+ 87,1
CD107+ 91,4
IFNG+ 82,3
TNF+ 74,9

B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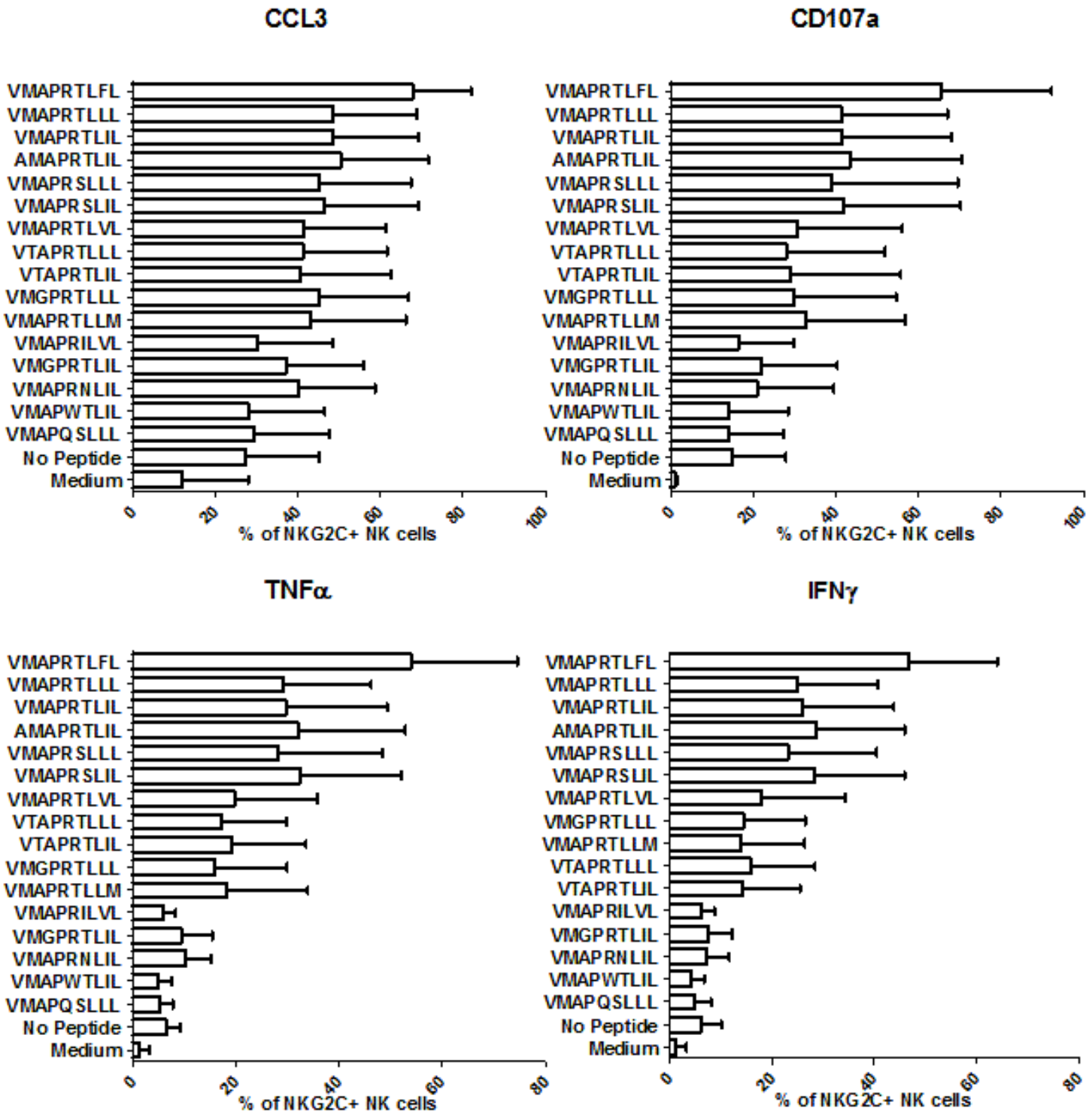
CD107a
IFNγ
TNFα
No peptide
VMAPWTLIL
VMAPRTLIL
VMAPRTLFL
CD56 PE Dazzle
CCL3 APC
CD107a Alexa488
IFNgamma PE-Vio770
TNF alpha BV605
CCL3+ 50,0
CD107+ 39,4
IFNG+ 9,64
TNF+ 7,01
CCL3+ 41,6
CD107+ 29,1
IFNG+ 6,97
TNF+ 2,62
CCL3+ 3,32
CD107+ 1,02
IFNG+ 0,59
TNF+ 0,23
CCL3+ 4,95
CD107+ 1,49
IFNG+ 0,46
TNF+ 0,15

C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VMGPRTLLL
VMAPRTLLM
VMAPRILVL
VMGPRTLIL
VMAPRNLIL
VMAPWTLIL
VMAPQSLLL
No Peptide
Medium
0
20
40
60
80
100
% of NKG2C+ NK cells
CD107a
VMAPRTLFL
VMAPRTLLL
VMAPRTLIL
AMAPRTLIL
VMAPRSLLL
VMAPRSLIL
VMAPRTLVL
VTAPRTLLL
VTAPRTLIL
VMGPRTLLL
VMAPRTLLM
VMAPRILVL
VMGPRTLIL
VMAPRNLIL
VMAPWTLIL
VMAPQSLLL
No Peptide
Medium
0
20
40
60
80
100
% of NKG2C+ NK cells
TNFα
VMAPRTLFL
VMAPRTLLL
VMAPRTLIL
AMAPRTLIL
VMAPRSLLL
VMAPRSLIL
VMAPRTLVL
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VMAPRILVL
VMGPRTLIL
VMAPRNLIL
VMAPWTLIL
VMAPQSLLL
No Peptide
Medium
0
20
40
60
80
% of NKG2C+ NK cells
IFNγ
VMAPRTLFL
VMAPRTLLL
VMAPRTLIL
AMAPRTLIL
VMAPRSLLL
VMAPRSLIL
VMAPRTLVL
VMGPRTLLL
VMAPRTLLM
VTAPRTLLL
VTAPRTLIL
VMAPRILVL
VMGPRTLIL
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Medium
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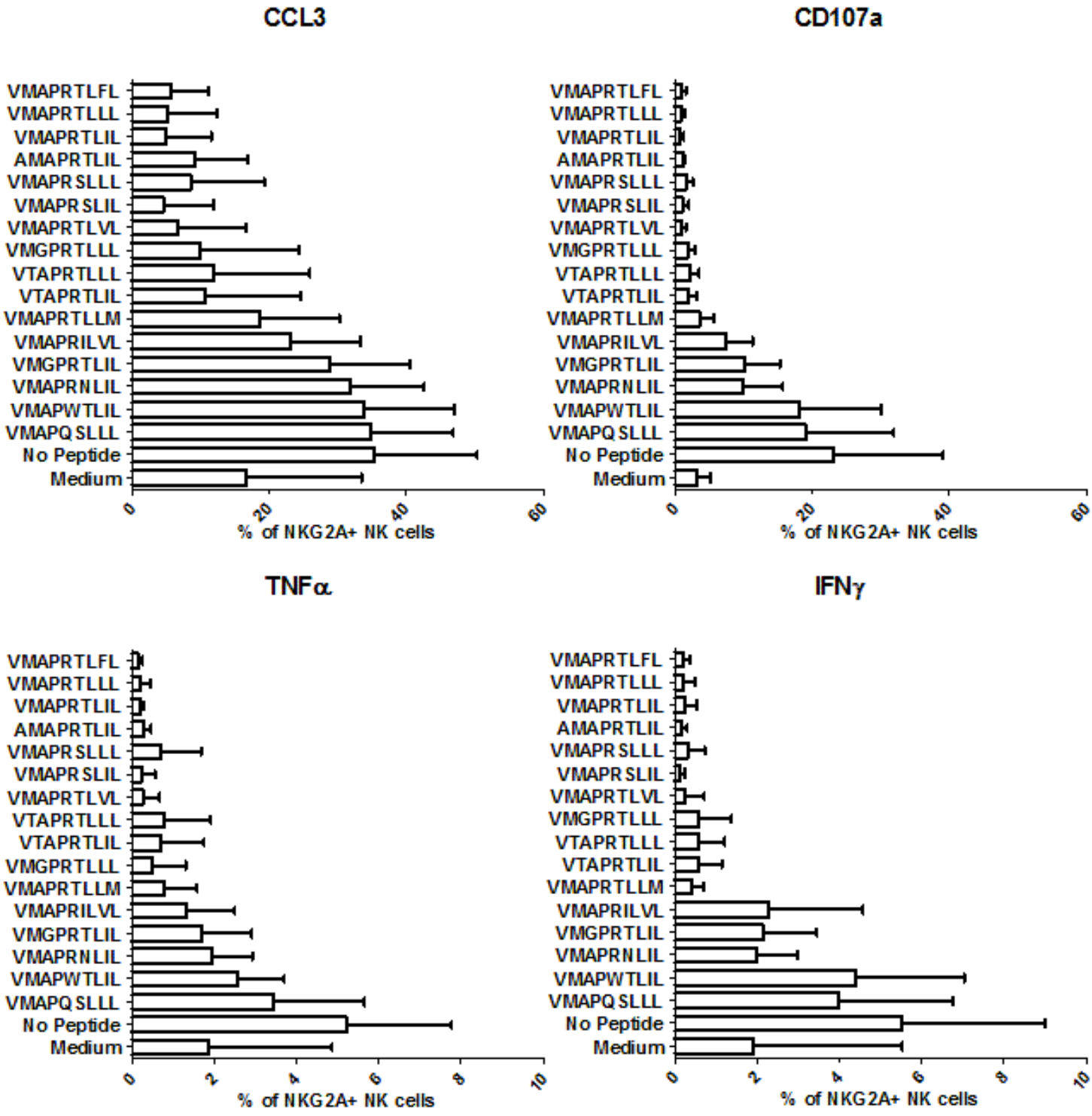
20
40
60
80
% of NKG2C+ NK cells

D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VTAPRTLIL
VMAPRTLLM
VMAPRILVL
VMGPRTLIL
VMAPRNLIL
VMAPWTLIL
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Medium
0
20
40
60
0
2
4
6
8
10
% of NKG2A+ NK cells

E

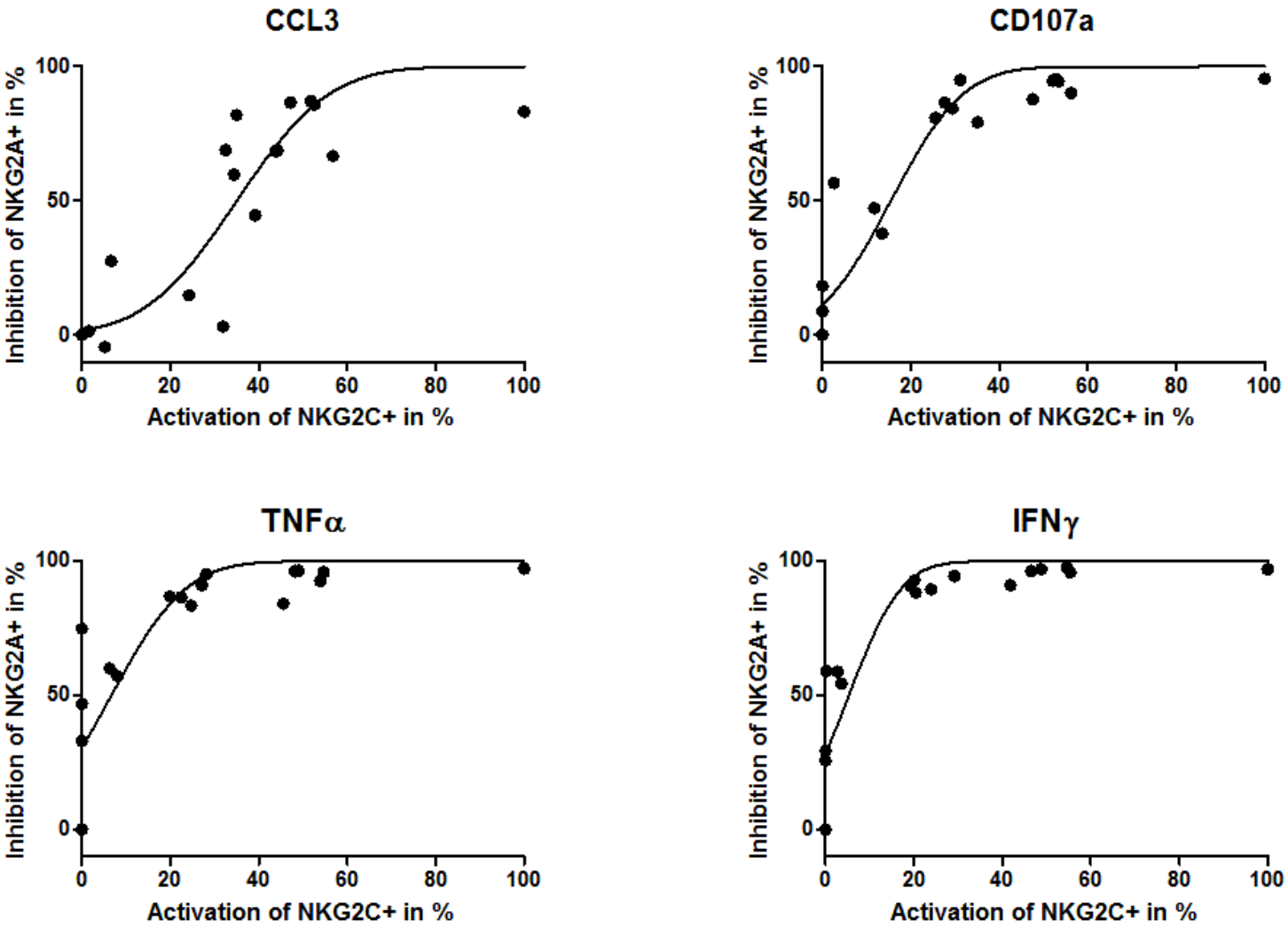


Figure 6: CMV UL40 derived peptides show different interaction potentials with NK cells and NKG2A+ NK cells are more sensitive than NKG2C+ NK cells (**A**) Representative FACS plot showing expression of different activation markers after incubation with peptide-pulsed K562/E. Gated on NKG2C+ NK cells and (**B**) NKG2A+ NK cells. (**C**) Summary of data with NK cell stimulation assays from 6 different donors in 3 different experiments with mean and standard deviation of activation marker positive NK cells of NKG2C+ NK cells and (**D**) NKG2A+ NK cells. (**E**) Activation of NKG2C+ NK cells plotted against the Inhibition of NKG2A+ NK cells for the 15 different peptides tested and regression curve derived for a cumulative Gaussian distribution. The values represent the mean of results from the six different donors. Result for stimulation without peptide is plotted at x=0;y=0
Activation: (activation with peptide - activation with no peptide) / activation with VMAPRTLFL
Inhibition: 1 - (activation with peptide / activation with no peptide)

Group	Peptide sequence
Strongly interacting	VMAPRTLFL
Intermediately interacting	VMAPRTLIL VMAPRTLLL VMAPRSLIL VMAPRSLLL AMAPRTLIL
	VMAPRTLVL VMAPRTLLM VTAPRTLIL VTAPRTLLL VMGPRTLLL
Not/weakly interacting	VMGPRTLIL VMAPR**N**LIL VMAPR**I**LVL VMAP**W**TLIL VMAP**Q**SLLL

Strength of interaction with NKG2C+ NK cells

Table 4: Grouping of peptides into different classes according to interaction potential

3.4 Expanded NKG2C+ NK cells in alloHSCT patients with CMV reactivation show a specific phenotype, which is influenced by CMV strains encoding for different leader peptides

Out of the 34 patients with CMV reactivation and known sequence of the CMV UL 40 leader peptide sequence we were able to analyze NK cells by FACS from 15 patients after CMV reactivation.

We further tested 6 patients with Recipient and Donor CMV-negative serostatus (R-D-), which should not have been in contact with CMV, as a control for the general influence of CMV on NK cells (**Table 5**). The patients were grouped according to the different peptides encoded by CMV.

One patient with an intermediately interacting peptide had a coinfection with a CMV strain encoding for LMAPRTLVL, which had not been tested, and one patient with the strongly interacting peptide VMAPRTLFL had a coinfection with a CMV strain encoding for the intermediately interacting peptide VMAPRTLLL. Two other patients had coinfection with other CMV strains, but both from the intermediate class (VMAPRTLIL + VMAPRTLLL and VMAPRTLLL + VMAPRTLVL). **Figure 7A** shows the distribution of the CMV strains encoding for the different peptides from the 15 patients.

To assess the influence of different CMV strains on NK cells we applied markers which are typically associated with adaptive NK cells. Typically the NKG2C+ expansion is associated with an increased frequency of CD2+ cells and a decreased frequency of Siglec-7+, NKG2A+ and FcεR1γ+ inside the expanded cells.[10,12,17,76] **Figure 7B** illustrates two examples; one is obtained from a patient with R-D- serostatus without expansion and the other from a patient with CMV reactivation and a very strong expansion of NKG2C+ NK cells showing the typical pattern of phenotypical markers.

Patients with CMV reactivation exhibit the expected increase of NKG2C+ NK cells compared to Recipient/Donor serostatus negative patients (**Figure 7C**). The patient with the not/weakly interacting peptide VMAPRNLIL had a higher proportion of NKG2C+ NK cells than the patients with the strongly interacting peptide VMAPRTLFL, suggesting an independency of %NKG2C+ NK cells from peptide properties.

By assessing typical markers of expanded NK cells inside the NKG2C+ and NKG2C- NK cell population and by considering the properties of the leader peptides encoded by different CMV strains, one can see which phenotypic changes are dependent on different peptides and NKG2C (**Figure 7D**). The frequency of Siglec-7+ cells was significantly lower in NKG2C+ and NKG2C- NK cells and showed no dependency on stronger interacting peptides, speaking for a NKG2C/peptide-independent induction of Siglec7 negativity. The frequency of CD2+ cells showed a trend towards higher frequency in NKG2C+ NK cells in patients with CMV reactivation, even though not significantly. As in the induction of Siglec-7- NK cells by CMV, the induction of CD2+ NK cells seems to be not dependent on high-affinity leader peptides encoded by CMV.

There is a non-significant trend towards a lower frequency of NKG2A+ NK cells inside NKG2C+ NK cells in patients with CMV reactivation. In contrast to CD2 and Siglec-7 the two patients infected with CMV strains encoding for the strongly interacting peptide were in this case the ones with stronger selection of NKG2A- NK cells inside the NKG2C+ NK cells. They had a much lower frequency than the patient infected with the not/weakly interacting peptide. These findings and the absence of the effect in NKG2C- NK cells suggest a dependency of the selection of NKG2A- NK cells on the NKG2C-peptide interaction.

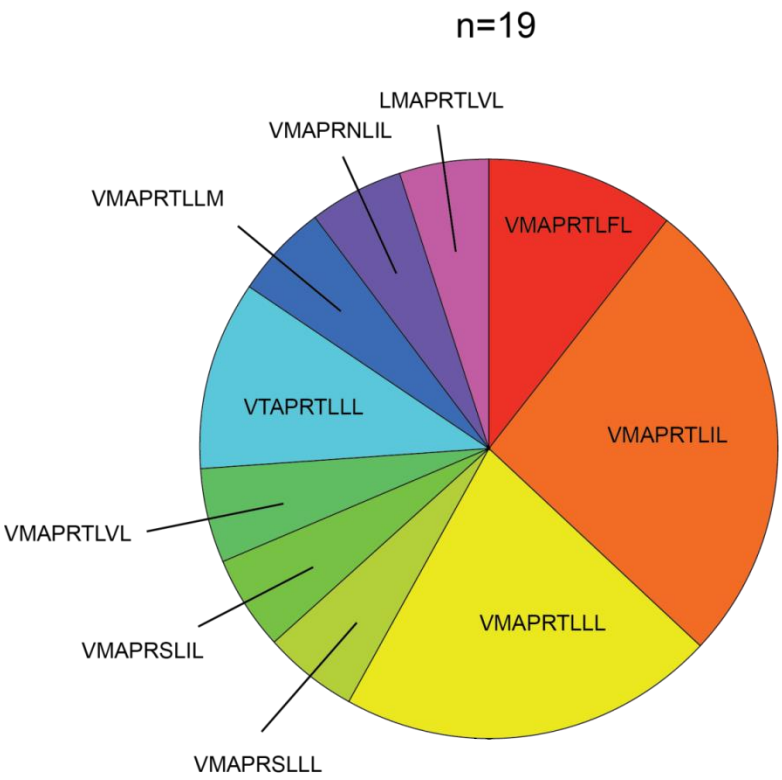
This NKG2C-peptide dependent effect was even more pronounced for FcεR1γ, where the selection of FcεR1γ- NK cells was much stronger in the two patients with the strongly interacting peptide and only present within NKG2C+ NK cell populations.

To better define expanded NK cells, we assessed NK cells coexpressing all 3 markers in the typical combination (CD2+, NKG2A- and Siglec7-), which were significantly increased inside the NKG2C+ and also inside the NKG2C- population of NK cells (**Figure 7E**). NKG2C+ NK cells with this specific combination of markers were slightly higher in frequency within the patients infected with a CMV strain encoding for the strongly interacting peptide, suggesting a dependency on the NKG2C-peptide interaction driven by selection of NKG2A- NK cells.

Variable	**CMV reactivation**	**CMV R-D- (controls)**
n	15	6
Age		
median	57	65,5
(range)	(21-72)	(52-75)
Gender		
Female	8	2
Male	7	4
Sample day after transplantation		
median(range)	138(46-297)	68(18-153)
CMV peptide sequence		
Intermediately interacting	12	
double peak	3	
Strongly interacting	2	-
double peak	1	
Not/weakly interacting	1	
double peak	0	
Peak viral load in cop/mL		
median (range)	25.000 (<2000-302000)	-

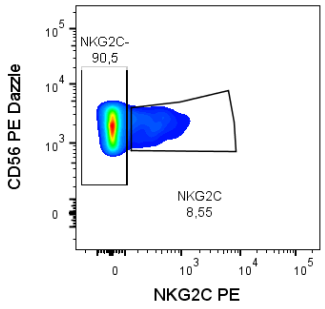
Table 5: Characteristics of patients with CMV reactivation and control patients with recipient and donor CMV-negative serostatus

A

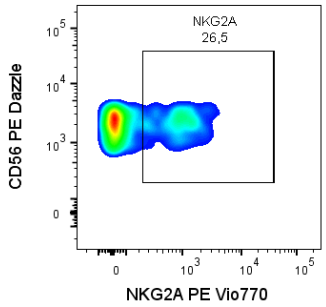


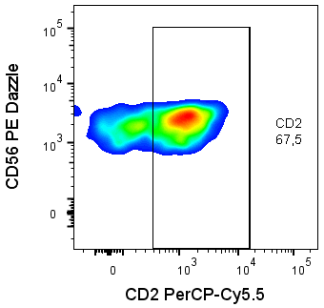
B

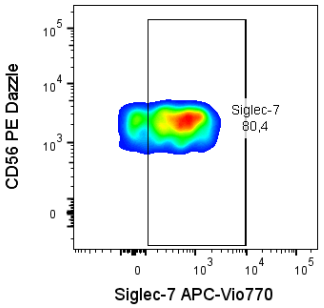
Patient with R-D- CMV serostatus

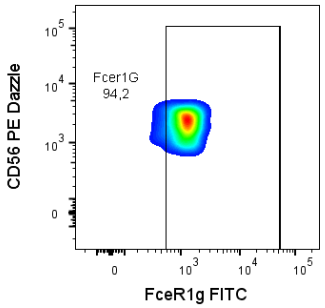


NKG2C+ NK cells

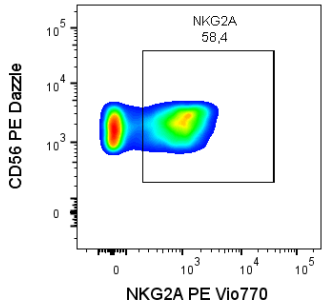


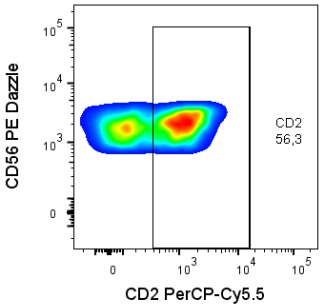


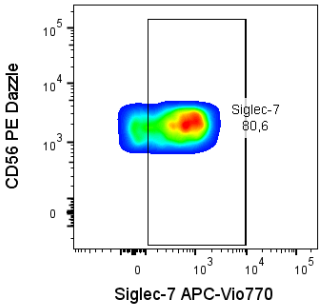


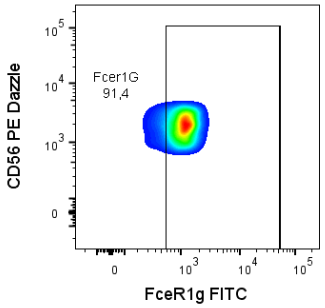


NKG2C- NK cells

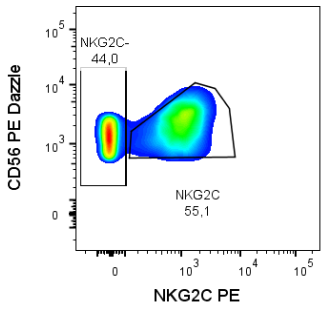




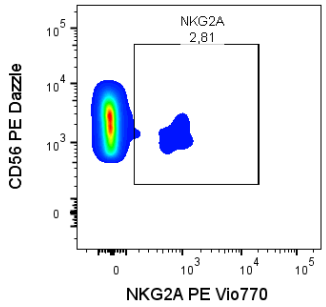


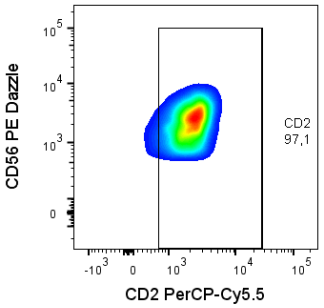


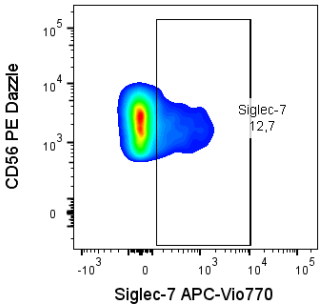
Patient with CMV reactivation

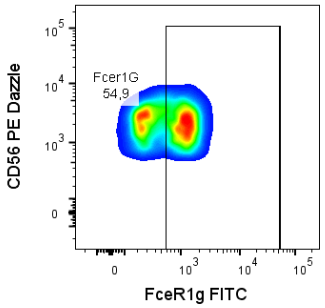


NKG2C+ NK cells

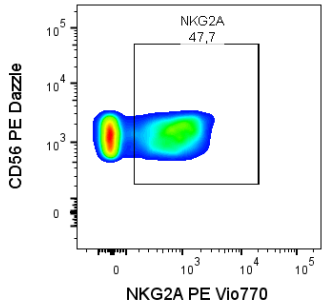


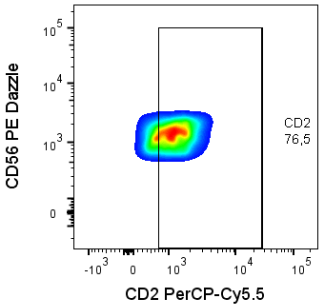


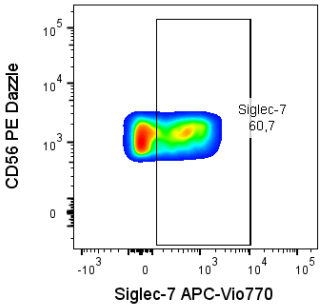


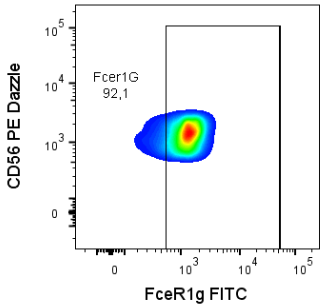


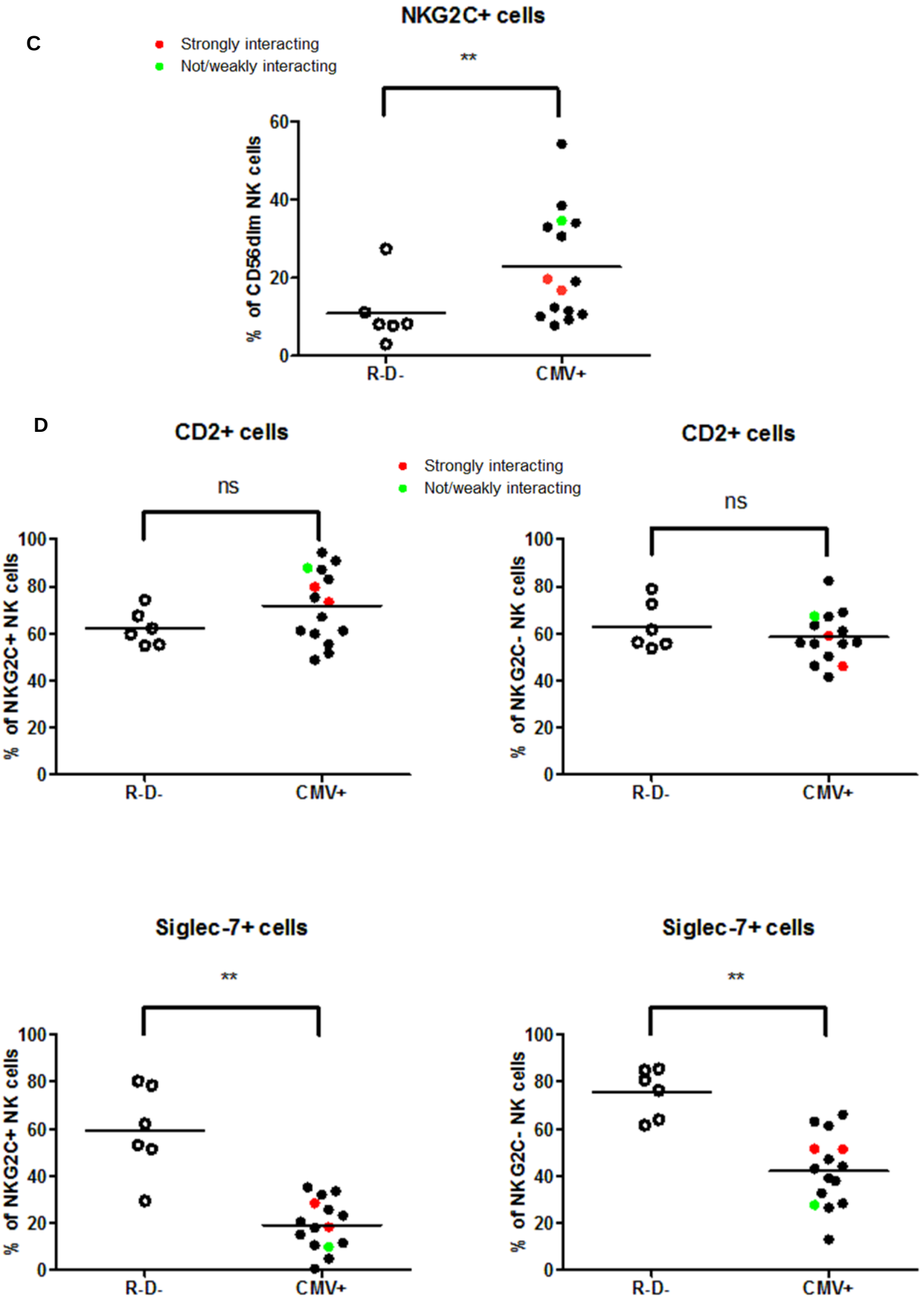
NKG2C- NK cells

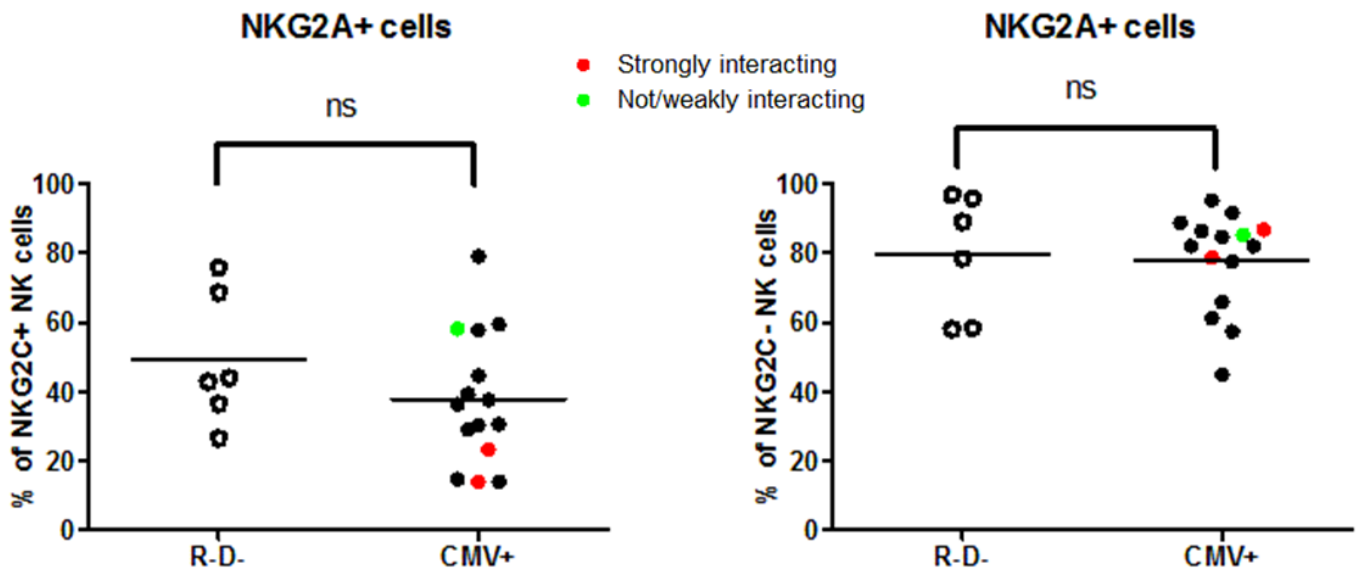


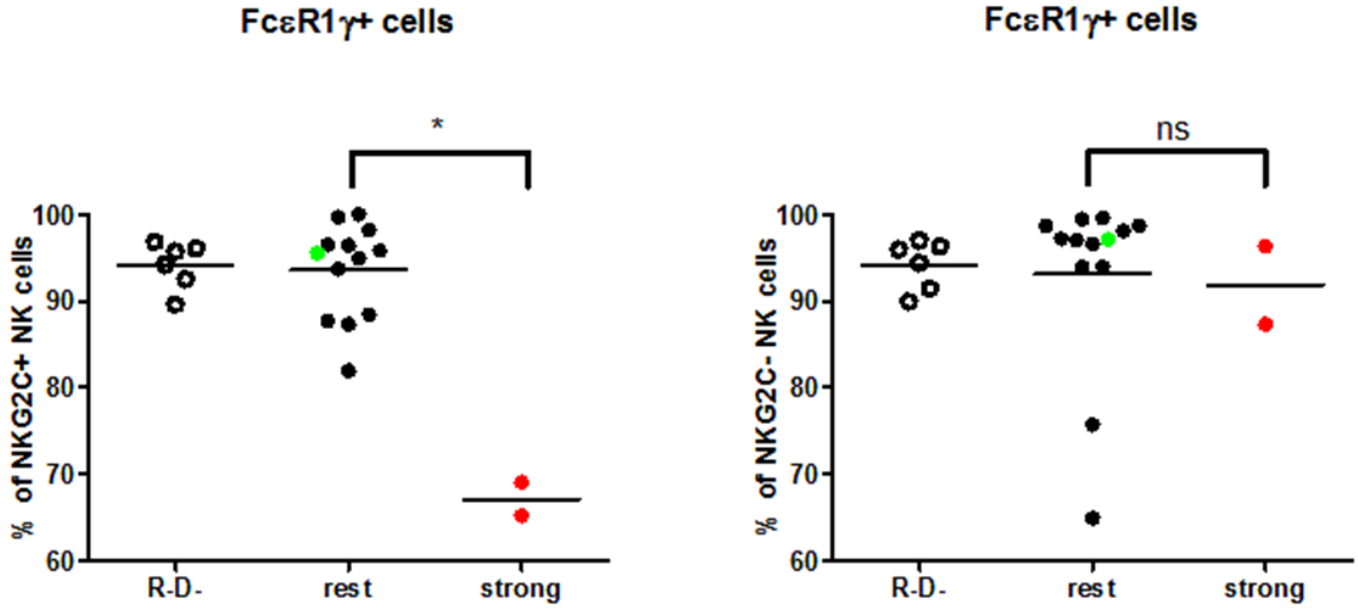







C
NKG2C+ cells
Strongly interacting
Not/weakly interacting
**
% of CD56dim NK cells
60
40
20
0
R-D-
CMV+
D
CD2+ cells
ns
Strongly interacting
Not/weakly interacting
% of NKG2C+ NK cells
100
80
60
40
20
0
R-D-
CMV+
CD2+ cells
ns
% of NKG2C- NK cells
Siglec-7+ cells
**
% of NKG2C+ NK cells
Siglec-7+ cells
**
% of NKG2C- NK cells


NKG2A+ cells
Strongly interacting
Not/weakly interacting
ns
% of NKG2C+ NK cells
100
80
60
40
20
0
R-D-
CMV+
NKG2A+ cells
ns
% of NKG2C- NK cells
100
80
60
40
20
0
R-D-
CMV+


FcεR1γ+ cells
*
% of NKG2C+ NK cells
100
90
80
70
60
R-D-
rest
strong
FcεR1γ+ cells
ns
% of NKG2C- NK cells
100
90
80
70
60
R-D-
rest
strong

E

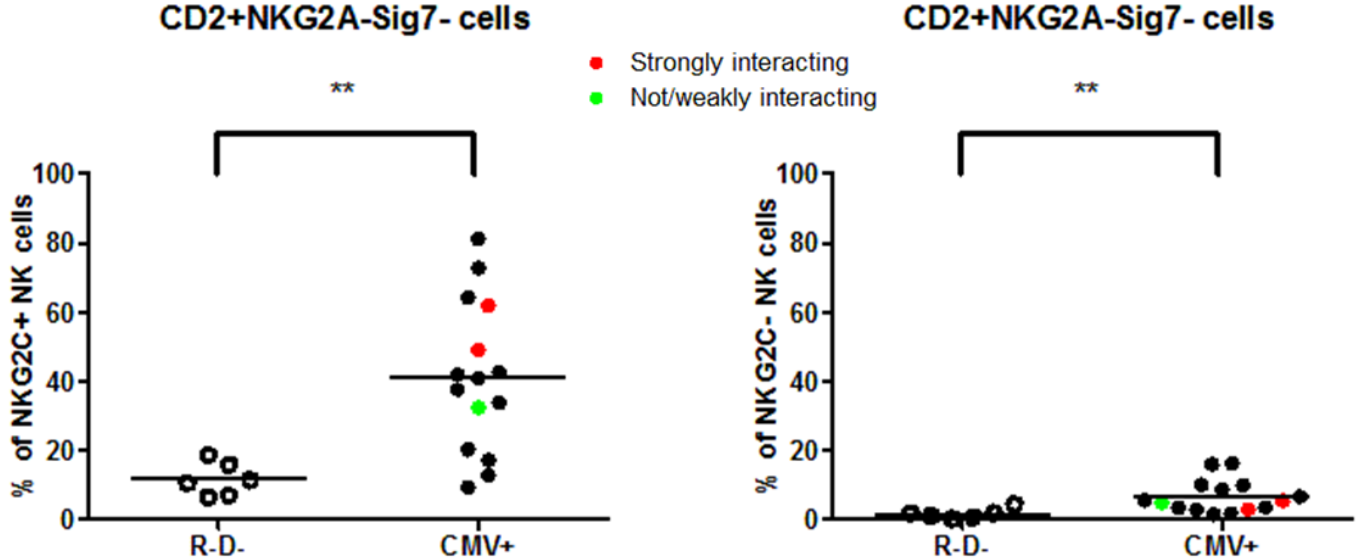

CD2+NKG2A-Sig7- cells
Strongly interacting
Not/weakly interacting
**
% of NKG2C+ NK cells
100
80
60
40
20
0
R-D-
CMV+
CD2+NKG2A-Sig7- cells
**
% of NKG2C- NK cells
100
80
60
40
20
0
R-D-
CMV+

Figure 7: The infection of patients with CMV strains encoding for UL40-derived leader peptides with differing interaction potentials impacts on the phenotype of NK cells. (**A**) Distribution of CMV UL 40 encoded leader peptides within the patients with FACS analysis of NK cells derived from Sanger sequencing. If the patient had a coinfection with another CMV strain, then both peptides were included. (**B**) Representative FACS plots of one patient with recipient/donor CMV negative serostatus (R-D-) and a patient with CMV reactivation and a very strong expansion of NKG2C+ NK cells displaying typical phenotypic changes. (**C**) Percentage of NKG2C+ NK cells from R-D- patients (n=6) and patients with CMV reactivation (n=15). (**D**) Expression of different phenotypic markers on NKG2C+ and NKG2C- NK cells comparing R-D- patients and patients with CMV reactivation. FcεR1γ+ NK cells are also compared between patients with an CMV strain encoding for a strongly interacting peptide (strong) and other patients with CMV reactivation (rest) (**E**) Comparison of NK cells with typical phenotypic coexpression pattern (CD2 positive; NKG2A negative; Siglec-7 negative) between patients with CMV reactivation and R-D- patients.

3.5 NK cells with typical coexpression pattern contribute to the NKG2C+ NK cell expansion

NK cells expressing the specific coexpression were the cells which contributed most to the expansion of NKG2C+ NK cells, when comparing R-D- patients and patients with CMV reactivation (**Figure 8**). Also, within the proportion of patients with CMV reactivation, a higher amount of CD2+, Siglec-7-, NKG2A- NK cells correlated with a higher overall size of the NKG2C+ NK cell expansion.

The patients with the strongly interacting peptide showed a stronger selection of the phenotype in relation to the overall frequency of NKG2C+ NK cells compared to the not/weakly interacting peptide, which could mean that the peptides are more important for the phenotype of the NKG2C+ expansion than the size.

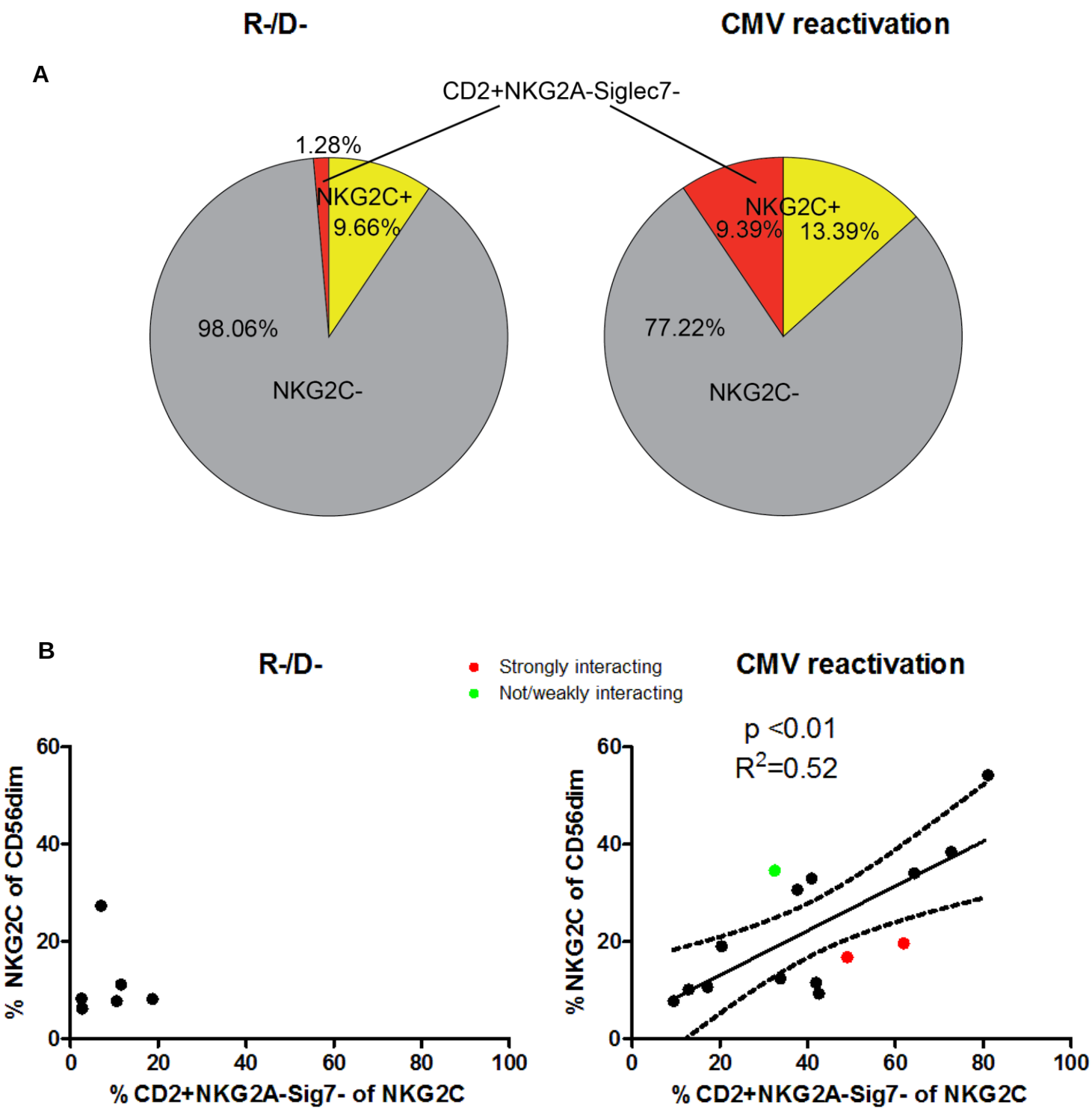


Figure 8: NK cells with a specific coexpression pattern are responsible for NKG2C+ NK cell expansion. (**A**) Pie chart demonstrating the distribution of NKG2C+ NK cells (red and yellow) and NKG2C+ NK cells with specific coexpression pattern (red) in patients with CMV R-D- serostatus (n=6) and patients with CMV reactivation (n=15). Data used are the mean values from the different patient groups. (**B**) Correlation of NK cells displaying a specific coexpression pattern (CD2 positive; NKG2A negative; Siglec-7 negative) with percentage of NKG2C+ NK cells within CD56dim NK cells. There is no correlation within R-D- patients.

3.6 NK cell expansion correlates with time after transplantation, but not with the monocyte count

The samples we obtained from our cohort were taken at different time points after transplantation. This was due to differing time periods of CMV reactivation after transplantation, but also due to the early mortality of patients in our cohort, which prevented further follow-up. **Figure 9** shows the influence of the time post transplantation on the frequency of NKG2C+ NK cells. There is a significant correlation with the overall size of the expansion, but not with the phenotype of the expansion, defined by the coexpression pattern. The samples from the two patients with the strongly interacting peptide, however, were obtained from earlier and later time points compared to the patient with the weakly interacting peptide, which makes a bias caused by time dependency unlikely.

Cichocki et al. have recently reported an association of higher monocyte counts with higher amount of NKG2C+ NK cells.[67] However, in our cohort we did not observe a correlation between the percentage of NKG2C+ NK cells and the monocyte count.

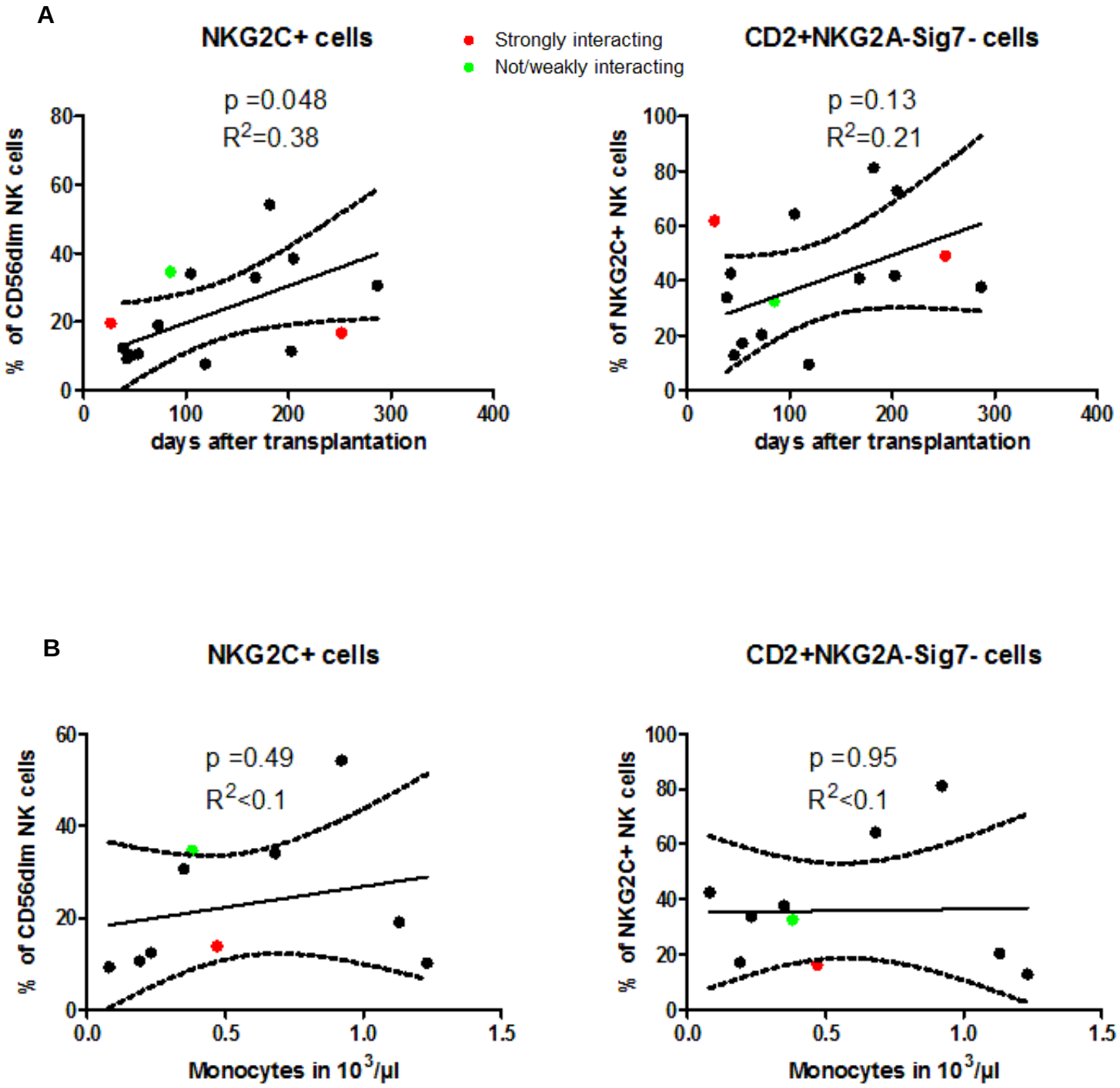


Figure 9: Percentage of NKG2C+ NK cells correlates with the time after transplantation, but not with the monocyte count. (**A**) Correlation of time after transplantation with percentage of NKG2C+ NK cells and NK cells with specific coexpression pattern of phenotypic markers in patients with CMV reactivation (n=15) and (**B**) correlation with absolute monocyte count

3.7 The adaptive phenotype of the NK cell expansion is associated with an improved clinical outcome

After investigating the effect of the different peptide classes on the selection and the phenotype of the NKG2C+ NK cell expansion, we also wanted to know if the markers of expansion could have any impact on the clinical outcome in patients with CMV reactivation (**Figure 10**).

The overall size of expanded NKG2C+ NK cells was not significantly higher in patients surviving without relapse, even though there was a trend towards a higher frequency of NKG2C+ NK cells. However, the patients which survived without relapse showed a significantly higher amount of NK cells coexpressing the typical markers of expanded NK cells and a significantly lower frequency of FcεR1γ+ cells.

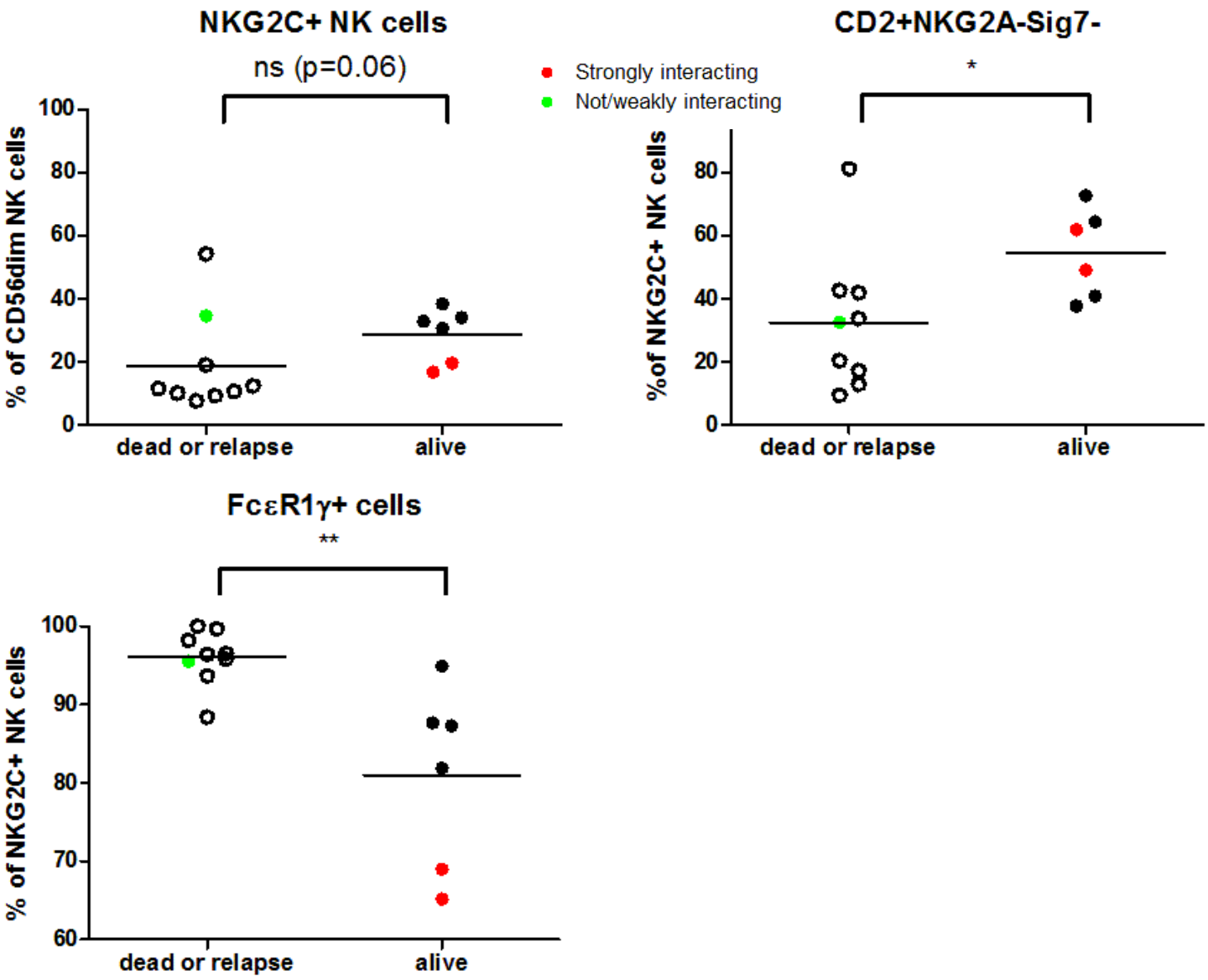


Figure 10: Patients surviving without relapse have a stronger induction of expanded NK cells. Comparison of percentage of NKG2C+ NK cells, specific coexpression pattern (CD2 positive; NKG2A negative; Siglec-7 negative) and FcεR1γ+ NK cells between patients that died later on or relapsed (n=9) and patients that survived without relapse (n=6)

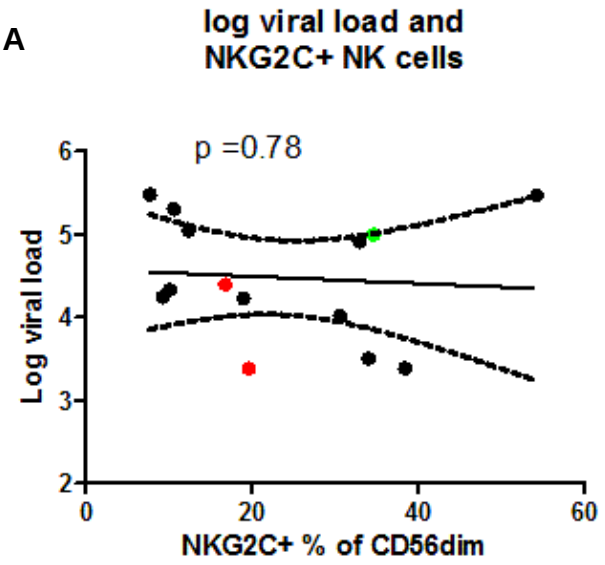
3.8 The stringency of monitoring CMV in alloHSCT patients has an impact on the strength of the reactivation, and expanded NK cells correlate with a lower peak viral load

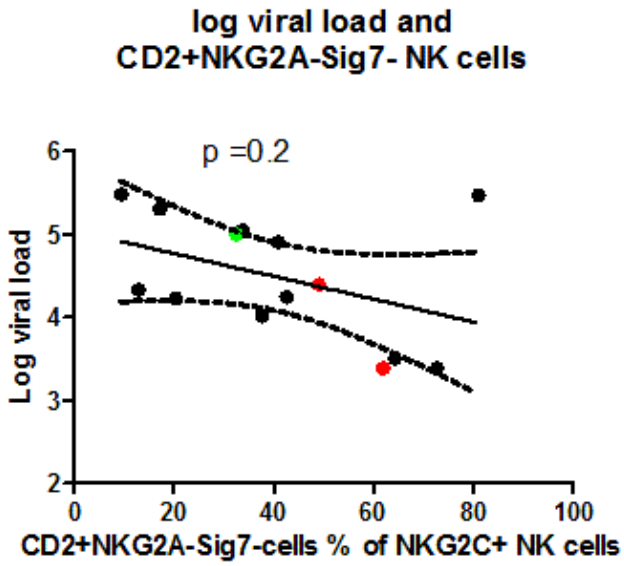
No correlation between the percentage of total NKG2C+ NK cells or of NKG2C+ displaying an adaptive phenotype with the peak viral load could be observed. (**Figure 11A**).

Normally, the patients were monitored weekly by qPCR for CMV reactivation. Patients who were not monitored regularly had a delay of diagnosis and consequently a higher viral load (**Figure 11B**), probably because of a later initiation of antiviral treatment. In line with this, the time from last qPCR control to diagnosis correlated with the viral load.

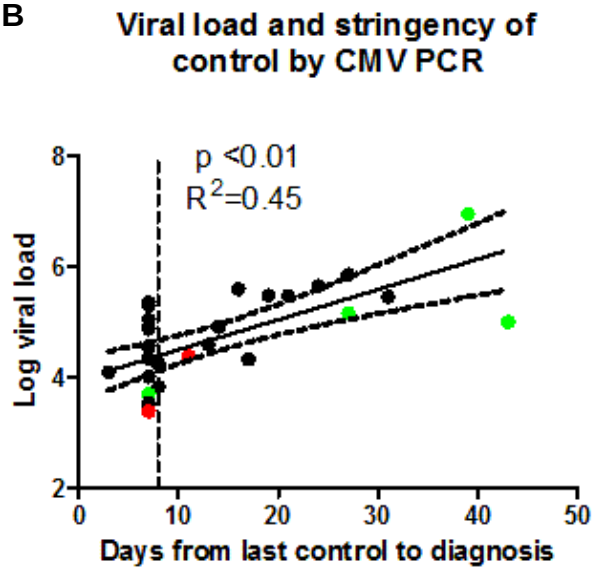
When selectively taking those patients into account who were monitored regularly and thus treated adequately, the percentage of NKG2C+ NK cells or of NKG2C+ displaying an adaptive phenotype correlated negatively with the viral load (**Figure 11C)**. This means that patients with a strong CMV reactivation did not have a strong expansion of adaptive NK cells, suggesting that patients with a robust expansion of NKG2C+ NK cells or of NKG2C+ NK cells displaying an adaptive phenotype might be able to better control the virus, at least when properly treated with antiviral drugs.

A

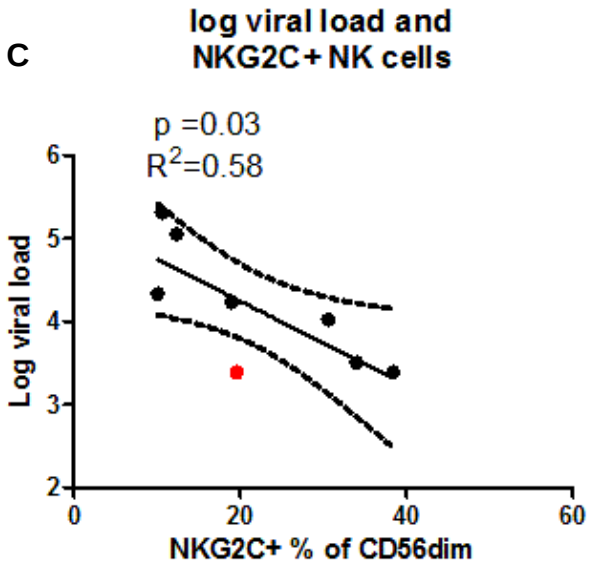

log viral load and
NKG2C+ NK cells
p =0.78
Log viral load
NKG2C+ % of CD56dim


log viral load and
CD2+NKG2A-Sig7- NK cells
p =0.2
Log viral load
CD2+NKG2A-Sig7-cells % of NKG2C+ NK cells

B


Viral load and stringency of
control by CMV PCR
p <0.01
R2=0.45
Log viral load
Days from last control to diagnosis

C


log viral load and
NKG2C+ NK cells
p =0.03
R2=0.58
Log viral load
NKG2C+ % of CD56dim

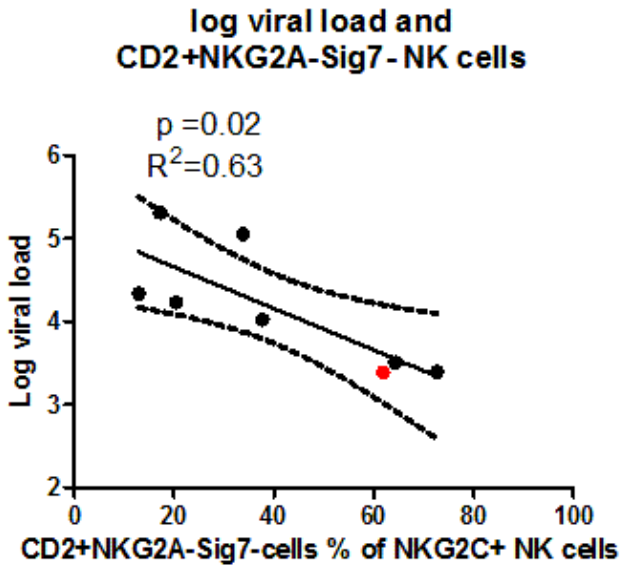

log viral load and
CD2+NKG2A-Sig7- NK cells
p =0.02
R2=0.63
Log viral load
CD2+NKG2A-Sig7-cells % of NKG2C+ NK cells

Figure 11: Patients with a stronger NKG2C+ NK cell expansion have lower viral loads during CMV reactivation (**A**) Correlation of peak viral load during CMV reactivation with percentage of NKG2C+ NK cells and NK cells showing a specific coexpression pattern (CD2 positive; NKG2A negative; Siglec-7 negative). All patients with FACS data and CMV > 2000 copies/ml were included (n=14) (**B**) Correlation of days from last negative CMV PCR to diagnosis with viral load. All patients with available data were included (n=31). Dashed line shows limit for inclusion for further analysis (only patients who were monitored weekly) (**C**) Correlation of peak viral load during CMV reactivation with percentage of NKG2C+ NK cells and NK cells showing a specific coexpression pattern (CD2 positive; NKG2A negative; Siglec-7 negative). Only patients that were monitored weekly are included. (n=8)

4 Discussion

As mentioned before, it became more and more clear in recent years that defined NK cell subsets display adaptive properties. The best characterized adaptive NK cell subset in humans is composed of NKG2C+ cells, which expand in response to CMV infection/reactivation.

Even though different receptor-ligand pairs have been shown to be potentially involved in mediating activation of NK cells by CMV infected cells, it is not known which interactions are most crucial for the development of adaptive NK cells and viral control. Probably there are several and also redundant pathways involved, which collectively lead to all the changes that are described for the NKG2C+ NK cell expansion. One should note that even NKG2C is dispensable for the expansion of adaptive NK cells[10].

Nevertheless, we chose to investigate the interaction of CMV and expanded NK cells by the axis of CMV UL40 encoded signal peptide/HLA-E and the NK cell receptor NKG2C/D94, because this receptor was the first to be described as being involved in human adaptive NK cells[9], the interaction is extensively characterized on a molecular basis[75] and it is the most frequent adaptive population in NK cells.[10]

In contrast to other studies addressing this peptide-NKG2C interaction, we here, for the first time, describe consequences of this interaction in vivo.

4.1 CMV UL 40 sequencing

In a recent study[33] Heatley et al. described the CMV UL40 gene polymorphism in a cohort of HSCT patients. The characterization of peptide polymorphism in our cohort is consistent with the results from their study. After repeatedly detecting double peaks in Sanger sequencing reads of some patients, we could show, by cloning of PCR products, that this event relates to the occurrence of mixed PCR products, likely reflecting mixed CMV sequences in vivo.

About 20% of the patients from our cohort showed such mixed CMV sequences. Moreover, we have identified new UL40 sequences, further supporting the large diversity displayed by CMV strains at this site. The CMV strains we sequenced encoded most commonly for peptides with an intermediate interaction potential (e.g. VMAPRTLIL), suggesting an evolutionary advantage of these variants, probably caused by host immune pressure.

The high frequency of mixed strain CMV infections seems somewhat unexpected, considering that CMV is a large DNA virus possessing a high fidelity DNA polymerase with 3'-5' exonuclease activity[77]. Recent deep sequencing studies on HMCV, however, have also shown such high intrahost variability, reaching an intrahost nucleotide diversity which is comparable to that of highly polymorphic RNA viruses, such as HIV.[78] Possessing a proofreading DNA polymerase, the mechanisms of this high diversity are probably different from those in RNA viruses. This is supported by our observation that samples taken at different points in time, but from the same patients, almost exclusively showed the same sequencing result, speaking against short term spontaneous mutations as the most important mechanism. Combined with the fact that CMV is capable of DNA recombination[79,80], the most reasonable explanation is therefore the occurrence of multiple strain infections. The occurrence of different CMV variants in one host could be considered as equivalent to the possession of different variants of NK cell receptors in the host.

If the human NK cells can have the activated NKG2C+ and the inhibited NKG2A+ NK cells, also different virus populations could produce peptides with different properties (e.g. in a patient acquiring a very strong expansion of NKG2C+ NK cells, a second virus population encoding for a peptide with a weaker interaction potential could be advantageous). One of our patients with a mixed strain infection and a strong NKG2C+ NK cell expansion showed only one sequence encoding for a weaker interacting peptide during a later second CMV reactivation, which could be an indication to support this hypothesis.

4.2 HLA-E stabilization by different peptides

The different peptides showed differences in their ability to stabilize HLA-E in a transfected cell line. Even though the analysis of our patient mainly cohort resulted in CMV UL40 encoded peptides different from those found by Heatley et al.[33], overlapping peptides were also tested, showing comparable results. Also, in our experimental setting, the peptide VMGPRTLIL was the peptide showing the weakest stabilization of HLA-E. Interestingly, the peptide VMGPRTLLL, which Heatley et al. did not test, induced a stronger expression of HLA-E. Other peptides with L instead of I at position 8 (e.g. VMAPRTLIL/VMAPRTLLL or VMAPRSLIL/VMAPRSLLL) showed no such big differences in the ability to induce HLA-E expression. With our data, it thus seems difficult to tell if there are certain positions of the peptide which are necessary for the stabilization of HLA-E, as combinations of several position changes can lead to differences in the ability to stabilize HLA-E.

One should note that we only tested the ability of the different peptides, but not the virus itself, to stabilize HLA-E. As mentioned previously, CMV inhibits the TAP-dependent processing of peptides inside infected cells.[36] To maintain the expression of the UL40-derived signal peptide, the virus must provide a TAP-independent mechanism of peptide processing. In their study, Prod'homme et al.[46] have nicely shown that this mechanism is facilitated by gpUL40 itself and that HLA-E stabilization is impaired without the N-terminus of the peptide. Probably also the sequence of the signal peptide itself could influence the processing, as the binding to the ER membrane or affinity to the signal peptide peptidase could be influenced by this sequence.[46]

With our model, it was not possible to determine the effect of the processing ability, because we already used the readily processed nonameric peptides.

4.3 Influence of different peptides on activation and inhibition of NK cells

We found different reactions of NK cells expressing certain NKG2-receptors, a possible (and strongly simplified) in vivo model is shown in **Figure 12**.

More importantly, we found profound differences in the activation and inhibition of NK cells by the different peptides, showing that NKG2C+ NK cells are differentially activated by different peptides. Interestingly, most CMV strains found in our cohort encoded for peptides which were able to nearly completely inhibit the response of NKG2A+ NK cells, but just intermediately activate NKG2C+ NK cells. **Figure 1** shows a possible model of the in vivo reaction of NK cells expressing certain NKG2/CD94 receptors. Still some patients were infected with virus strains encoding for peptides with other properties, which could suggest a superior immunoevasion of certain virus strains in specific host situations. As there is a considerable number of individuals with a homozygous NKG2C deletion (~4%)[10], a virus strain encoding for the peptide with the strongest activation potential VMAPRTLFL could have better immunoevasion properties in those persons. We found strains encoding for this peptide in 2 out of 34 patients. Neither of the two patients had NKG2C deficiency. Certainly much more patients have to be assessed to show an immunoevasion advantage of specific CMV strains in specific patient subgroups.

Our data shows that NK cells expressing the inhibitory receptor NKG2A are already maximally inhibited by stimulation with peptides which are recognized only with intermediate efficacy by NKG2C+ NK cells, suggesting that NKG2A might react more sensitively to HLA-E/peptide complexes.

Indeed, the difference in the sensitivity of those receptors corresponds to the higher affinity of HLA-E/peptide complexes to NKG2A compared to NKG2C.[73]

NK cells have the ability to display effector functions, such as release of CCL2, IFNγ or TNFα and degranulation (CD107a) in response to combined engagement of different activating receptors. Fauriat et al.[72] have previously determined the different thresholds of activation required to induce distinct effector functions in NK cells, e.g. CCL3<CD107a<TNFα<IFNγ, implying that induction of IFNγ is generally achieved only in the presence of multiple receptor engagement. Interestingly, our results show nearly similar thresholds in NKG2C+ NK cells when triggered via one single receptor (NKG2C) with peptides displaying different functional avidity.

Indeed, degranulation and the production of the chemokine CCL3 could be easily induced in NKG2C+ NK cells even by intermediate peptides, while high production of the cytokines IFNγ and TNFα was achieved only in the presence of the high avidity peptide.

In NKG2A+ NK cells, the baseline activation (activation without peptide) followed the same pattern as in NKG2C+ NK cells. While there were about 40% CCL3+ NK cells without any peptide, only about 6% were TNFα+. Not surprisingly, those markers with lower baseline activation are more easily inhibited to the full extent than those with higher baseline activations. Thus, the different threshold of inhibition of the different markers in NKG2A+ NK cells is in line with the threshold of activation of NKG2C+ NK cells (e.g. IFNγ has a high activation threshold in NKG2C+ NK cells, and baseline activation in NKG2A+ NK cells is fully inhibited already by lower affinity peptides).

The molecular basis for the interaction of the HLA-E/peptide complex and NKG2/CD94 receptors is well characterized.[73–75] Positions 5 and 6 of the nonameric peptide are thought to be important for the interaction with CD94 and position 8 for the interaction with NKG2A/C. Our data supports this; peptides having changes at position 5 or 6, compared to the most common peptide VMAPRTLIL, are also those with the biggest change in activation and inhibition of the respective NK cells. One exception is given by a change from T to S at position 6, which had no influence. Changes at position 8 had a stronger influence on the activation of NKG2C+ NK cells, which seem to have the ability to be fine-tuned by the polymorphism at position 8, while in NK cells expressing the high affinity receptor NKG2A, full inhibition is reached irrespective of the residue at position 8 in our model.

One should note, that we tested the activation/inhibition of NK cells at saturated peptide concentrations and that with a lower availability of peptide or target cells (or a higher baseline activation), which all might be present in an in vivo setting, the inhibition of NKG2A+ NK cells could probably also be fine-tuned.

We did not test the influence of a mixture of tumor cells pulsed with different peptides (which might be present in mixed strain infections) on the response of NK cells seen in some of our patients. Probably the mechanism of interaction between the different peptides would be more complicated than just being pure competition. Cassidy et al.[29] suggest mechanisms like “peptide antagonism” or “peptide synergy” between different peptides.

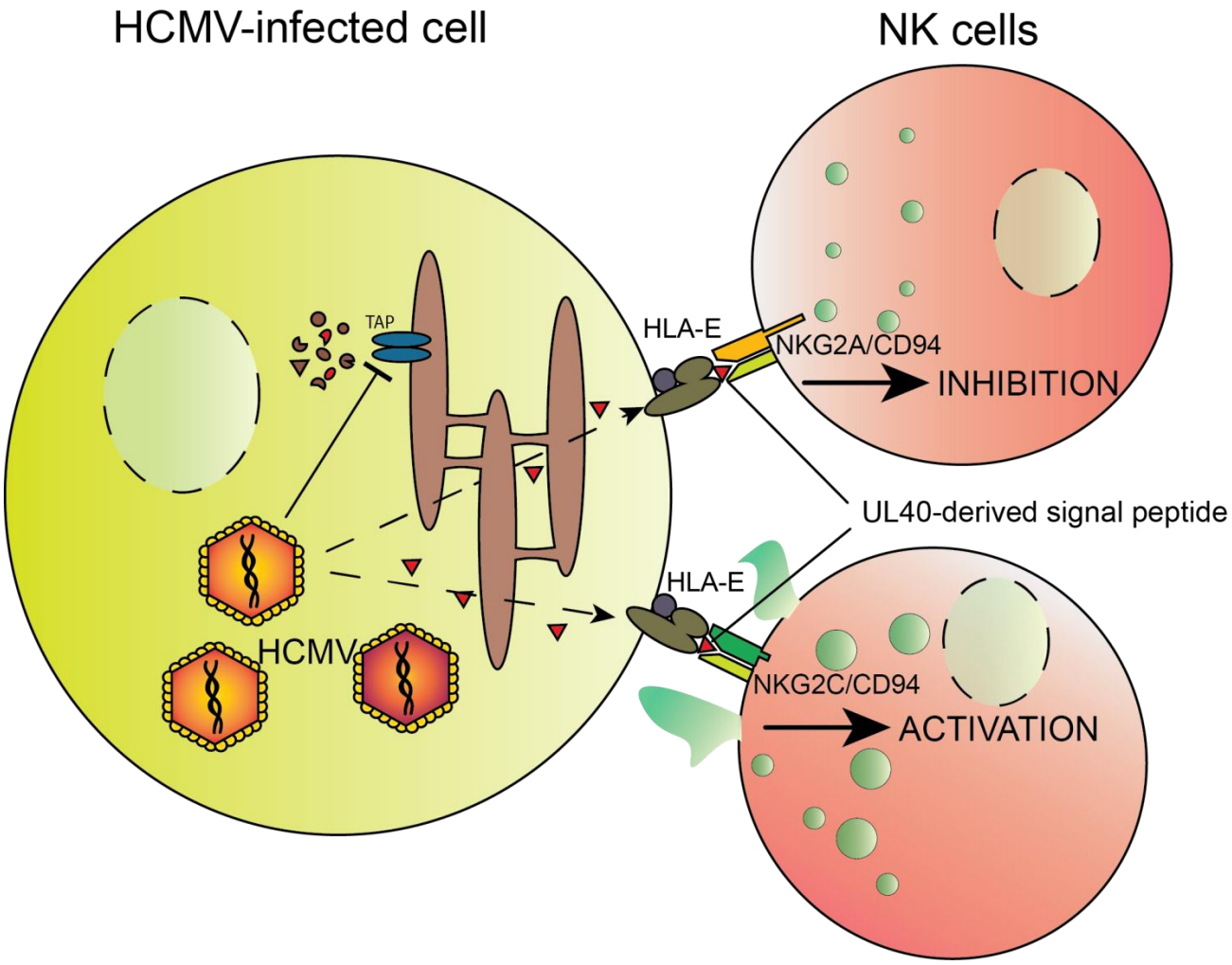


Figure 12: A speculative model which shows the immunoevasion of CMV by inhibition of CD94/NKG2A+ NK cells, while CD94/NKG2C+ NK cells are activated

4.4 Analysis of NK cells from alloHSCT patients

By analyzing phenotypic markers of NK cells in patients with known CMV UL40 leader peptide sequences, we could determine the influence of the encoded peptide on adaptive NKG2C+ NK cells. Even though we detected just a few patients with CMV strains encoding for peptides with highly different properties determined by our stimulation assay, the influences on some NK cell phenotypic markers were profound.

In our data set, the marker with the highest sensitivity to detect an exposure to CMV was Siglec-7, which is, however, independent on the peptide-NKG2C axis, as a much lower frequency of Siglec-7+ NK cells was observed both, in NKG2C+ and NKG2C- NK cells and there was no influence of the peptide avidity on the frequency of Siglec-7- NK cells. This suggests that this effect is caused by a different pathway, perhaps involving the glycocalyx, which is targeted by Siglec-7[81] as well as CMV[82]. In line with these data, down-regulation of Siglec-7 is not a hallmark of CMV but can also be found in other viral infections[83–85]. Also interestingly, Siglec-7 modulates NK cell response to tumor cells,[86] and it has been shown that T-ALL blasts could evade NK cell immunosurveillance by upregulation of Siglec-7 ligands.[87] Based on those facts, one could speculate that the lower frequency of Siglec-7 in patients with CMV reactivation could lead to a better immunosurveillance by NK cells.

The costimulatory molecule CD2 was also found to be independent from the peptide-NKG2C axis, and even though it is a marker associated with CMV-induced adaptive NK cells[10], it did not reach significance in our small cohort in comparison of patients undergoing or not undergoing reactivation with CMV.

As the costimulatory molecule CD2, which is also typically expressed on T cells, and its ligand CD58, are involved in many cell-cell interactions[88,89], it is not surprising, that the peptide-NKG2C axis does not lead to a specific induction. Perhaps there are more unspecific factors, e.g. inflammatory cytokines, involved in the induction of CD2 on NK cells. In line with this, Sun et al. have nicely shown the importance of IL-12 for the generation of adaptive NK cells in mouse cytomegalovirus.[90] Most likely, some changes observed in human adaptive NK cells could be attributed to this axis of interaction.

The selection of NKG2A- NK cells inside the NKG2C+ NK cells was found to be influenced by the peptide-NKG2C axis, even though not as strongly as FcεRIγ- NK cells were. Because NKG2A has the same ligands as NKG2C, one might think that the effect is caused independently of NKG2C (NKG2A+ NK cells could be negatively selected, because they are inhibited by the peptide/HLA-E complex). The fact that NKG2C- NK cells seem to have a peptide-independent pattern of NKG2A frequency, with nearly similar frequencies of NKG2A+ NK cells in the patients with the strongly and the not/weakly interacting peptides (we did not observe a negative selection of NKG2A+ NK cell inside the NKG2C- NK cells influenced by the peptide/HLA-E complex). Our data is in line with the hypothesis that the engagement of the NKG2C receptor is necessary for the selection of NKG2A- cells.

There was a very strong selection of FcεRIγ- NK cells within the NKG2C+ NK cells in the two patients with the strongly interacting peptide. Interestingly, some of our patients showed an accumulation of FcεRIγ- within NKG2C- NK cells, but not in those two with the strongly interacting peptide, where FcεRIγ- were confined to NKG2C+ ones. This shows that the peptide-NKG2C axis is not per se necessary for the induction of FcεRIγ- NK cells, but leads to a preferential accumulation of those cells in the NKG2C+ NK cell population. The change in the expression of the signaling adaptor FcεRIγ is thought to be a marker of a more profound epigenetic reprogramming of adaptive NK cells and it was suggested that signaling adaptor molecules in adaptive NK cells are responsible for functional differences.[12]

Taken together, the influence of the peptide-NKG2C axis on NK cells is much more complicated than just being: *higher affinity peptides mean more adaptive NK cells.* It rather seems to be the following: *different peptides mean adaptive NK cells with a different phenotype.* Adaptive NK cells can also be found in individuals with a deletion of the NKG2C locus and no influence of the peptide-NKG2C axis.[10] One could sum up our findings as follows: The engagement of NKG2C+ NK cells by stronger interacting peptide makes it more likely for adaptive NK cells to accumulate inside the compartment of NKG2C+ NK cells.

The profound induction of Siglec-7- NK cells within the whole population of NK cells in contrast to the mild induction of other markers in specific subsets shows that CMV does not just induce the generation of one adaptive, homogenous NK cell population, but does rather cause different changes in different cell population at the same time, this is very likely further complicated by the influence of spatiotemporal factors. Nevertheless, we found an influence of different peptides on the pattern of different markers within the different NK cell population, which can be considered as an adaptive feature of NK cells.

One important limitation of our study is given low number of patients analyzed, thus not all conclusions drawn are based on statistically significant results, but still, it is very unlikely, that different CMV UL40-encoded peptides have no influence on NK cells.

4.5 Other factors influencing the NK cell expansion

In order to check for possible biases of our data, we tried to look for other factors, which could influence the frequency and phenotype of NKG2C+ NK cells. As mentioned above Cichocki et al. reported an association of higher monocyte counts with higher amount of NKG2C+ NK cells.[67] We did not observe this correlation in our cohort, which is not surprising, as we examined a much smaller cohort, and the correlation that Cichocki et al. had observed, was not so strong (R^2=0.29). One important limitation of our study is that the samples obtained for the analysis of the NK cell phenotype were not gathered from the same time points after transplantation, on the other hand, we were able to obtain samples from patients with early mortality, which could not be included in recent studies investigating the phenotypic properties of CMV-induced NK cells after alloHSCT.[68]

The frequency of NKG2C+ NK cells correlated weakly with the time after transplantation, while no significant correlation could be observed with the amount of NK cells expressing the pattern typical for adaptive NK cells.

Even though a bias of our data concerning the time after transplantation cannot be fully excluded, the fact that the samples from the two patients with the CMV strain encoding for the strongly interacting peptide were from very different time points, but still showed similar phenotypic changes, makes it extremely unlikely that our data for the peptide-specific phenotype induction is biased by time.

4.6 Association of adaptive NK cells with improved clinical outcome

It has been reported that CMV reactivation after alloHSCT can lead to a lower relapse rate of the underlying disease[56]. Given the important role of NK cells in alloHSCT, it could be possible that NK cells are involved in the mediation of this virus-versus-leukemia effect.

Even though we have not found a lower relapse rate in patients with CMV reactivation in our small cohort, we wanted to look at the influence of the shaping of the NK cell repertoire by different CMV strains on the clinical outcome.

Patients who survived and had no relapse showed NKG2C+ NK cells expressing markers associated with adaptive properties. The markers which have been shown to be influenced by the peptide-NKG2C axis (NKG2A-, FcεRIγ-) were also associated with a better clinical outcome. Interestingly, the two patients with the CMV UL40 encoded strongly interacting peptide also survived without relapse for at least 1 year.

With just 15 patients included, it was of course not possible to perform a detailed multivariate survival analysis. There has not been any systematic follow-up of the patients, as the clinical data was obtained retrospectively.

Nevertheless, our data gives a hint for some association of a specific NK cell phenotype with a better clinical outcome, which could also be influenced by certain CMV strains. As it has been shown that HLA-E is expressed by most leukemia cell lines and by some other tumor cell lines[91], it is tempting to speculate that HLA-E recognition by NKG2C could be involved in mediating protection from malignancy.

It is thought that malignant cells maintain the HLA-E expression to inhibit NK cell lysis through NKG2A and indeed some in vitro and in vivo data showing improved NK cell mediated lysis after NKG2A inhibition, supports this hypothesis.[92,93] However, the lower frequency of NKG2A+ NK cells following infection by CMV, especially by strains with UL40-encoded strongly interacting peptide, could indirectly favor an improved lysis of leukemia cells by NK cells. Induction of adaptive NK cells by CMV itself could lead to higher functional capacities and better protection from infection and relapse.

In a study of Bjorklund et al.[68], it was shown that the induction of adaptive NK cells by CMV reactivation has been associated with a worse clinical outcome after alloHSCT. In contrast to our study, they obtained the patient samples for FACS analysis at 9-12 months after transplantation, while we have used samples from earlier time points. Patients with early relapse and death were thus not included in their study. Furthermore, they included all patients with at least donor or recipient positive CMV serostatus in their analysis, while we analyzed the NK cell phenotype inside the patients with detectable CMV reactivation and used patients with CMV R-/D- serostatus as controls. We also used a strategy to analyze the phenotype and predominantly concentrated on the NKG2C mediated effects, while Bjorklund et al. used a cluster analysis with different markers of the whole NK cell population, including the expression of educating KIRs.

Taken together, our data suggests that it could be valuable to further validate the correlation between NK cell repertoire shaping by infection with different HCMV strains and clinical outcome of alloHSCT patients. Developing strategies aiming at manipulating the peptide-NKG2C axis could have beneficial effects for both: NK cell-based tumor therapy and CMV-vaccination.

4.7 Correlation between low viral titer and adaptive NK cells

Even though the induction of adaptive NK cells by CMV is well-established, the role of those NK cells in the control of CMV remains to be further elucidated.

A very recent prospective study by Redondo-Pachon et al.[94] showed a significantly lower rate of CMV reactivation in kidney transplant recipients which had a higher amount of NKG2C+ NK cells before transplantation.

In our study, however, we did not investigate the properties of NK cells before the CMV reactivation to look for a protection, but investigated the NK cell phenotype after reactivation. Based on the data from Bjorklund et al.[68], which showed a stronger increase of NKG2C+ NK cells in patients with a more severe CMV reactivation, we expected such a dose effect of CMV in the induction of adaptive NK cells and consequently a correlation of a higher viral load with more adaptive NK cells after reactivation. Surprisingly, after excluding patients who were not monitored regularly by CMV PCR, we found the exact opposite, with more adaptive NK cells after CMV reactivation being associated with a lower viral load. When looking closely at the data from Bjorklund et al.[68], the stronger increase of NKG2C+ NK cells in patients with complicated CMV reactivation is mainly driven by a low frequency of such cells within the donor before transplantation and thus it does not contradict our findings.

Even though the correlation between a higher frequency of adaptive NK cells after CMV reactivation and a lower viral load does not prove any causal relationship, it is tempting to speculate that patients with a robust NK cell expansion were able to better control the CMV reactivation. We did not analyze the donor NK cells before transplantation and, because of low cell numbers, we did not have a chance to look at the recipient NK cells before reactivation. It is thus difficult to say if the transplanted NK cells[13] or the expansion inside the host contributed to the viral control. One interesting fact is that one patient with the strongly interacting peptide had a lower frequency of NKG2C+ NK cells and was still able to control the CMV reactivation.

Taken together, the lack of prospective data and also the concurrent antiviral treatment makes it difficult to use this data supporting the clinical role of NK cells in viral control, as previously suggested by Redondo-Pachon et al.[94].

4.8 Discussion of Hypothesis

We hypothesized that different CMV strains would have an influence on the properties of adaptive NK cells in an in vivo setting. Our data supports this hypothesis, as we found phenotypic differences of NK cells depending on the interaction potential of the CMV UL40-encoded peptide. Despite the low patient number analyzed and the low frequency of certain CMV strains, we were able to find some indications on the influence of viral control and improved clinical outcome.

Taken together, our study showed for the first time the influence of different CMV strains on the NK cell repertoire in alloHSCT patients. Upon validation in a larger cohort of patients, this finding could signal a clinical impact in patients undergoing alloHSCT.

Finally, one could speculate about possible therapeutic concepts derived from the data obtained. The strong reaction of some NK cells to certain peptides could be used for in vitro expansion of adaptive NK cells, which could then be used in a cell-based therapy, either for viral or leukemia control.

Another idea would be to use peptides or peptide-derived complexes in a vaccination strategy to expand adaptive NK cells in vivo, which also could possibly lead to a better control of CMV or leukemia.

Taken together, this work provides a solid starting point for further questions to be asked, as well as possible clinical applications in the future.

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Eidesstattliche Versicherung

„Ich, André Haubner, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: NK cells in patients undergoing allogeneic stem cell transplantation are influenced by the CMV UL40 gene polymorphism selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der zu Beginn stehenden gemeinsamen Erklärung mit der Betreuerin, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

André Haubner

Datum Unterschrift

Anteilserklärung an etwaigen erfolgten Publikationen:

André Haubner hatte Anteil an folgender Publikation:

Quirin Hammer, Timo Rückert, Eva Maria Borst, Josefine Dunst, André Haubner, Pawel Durek, Frederik Heinrich, Gilles Gasparoni, Marina Babic, Adriana Tomic, Gabriella Pietra, Mikalai Nienen, Igor Wolfgang Blau, Jörg Hofmann, Il-Kang Na, Immo Prinz, Christian Koenecke, Philipp Hemmati, Nina Babel, Renate Arnold, Jörn Walter, Kevin Thurley, Mir-Farzin Mashreghi, Martin Messerle and Chiara Romagnani.
Peptide-Specific Recognition of Human Cytomegalovirus Strains Controls the Activation and Expansion of Adaptive Natural Killer Cells. *Nat Immunol*. 2018;19(5):453-463. doi: 10.1038/s41590-018-0082-6.

Beitrag im Einzelnen:

- die Experimente und Analyse zur Akquirierung des größten Teils neuer CMV UL40-Sequenzen, welche in Figure 1 bzw. Supplementary Table 2 der genannten Publikation dargestellt werden
- die Experimente und Analyse von NK Zellen der stammzellstranplantierten Patienten in Figure 7 und Supplementary Figure 7 (zum größten Teil und ausgenommen Figure 7d und Suppl. Figure 7c)
- Akquirierung des größten Teils der klinischen Daten in Supplementary Table 3

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Unterschrift, Datum und Stempel der betreuenden Hochschullehrerin

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

List of publications

Quirin Hammer, Timo Rückert, Eva Maria Borst, Josefine Dunst, André Haubner, Pawel Durek, Frederik Heinrich, Gilles Gasparoni, Marina Babic, Adriana Tomic, Gabriella Pietra, Mikalai Nienen, Igor Wolfgang Blau, Jörg Hofmann, Il-Kang Na, Immo Prinz, Christian Koenecke, Philipp Hemmati, Nina Babel, Renate Arnold, Jörn Walter, Kevin Thurley, Mir-Farzin Mashreghi, Martin Messerle and Chiara Romagnani.

Peptide-Specific Recognition of Human Cytomegalovirus Strains Controls the Activation and Expansion of Adaptive Natural Killer Cells. *Nat Immunol*. 2018;19(5):453-463. doi: 10.1038/s41590-018-0082-6

Danksagung

Mein Dank gilt vor allem meiner Betreuerin Chiara Romagnani, welche mir eine Promotion in ihrer ambitionierten Arbeitsgruppe ermöglicht hat. Außerdem möchte ich allen anderen Mitgliedern der Arbeitsgruppe und insbesondere Quirin Hammer für die Betreuung und die konstruktive Kritik danken.

Ganz besonders möchte ich außerdem meiner Frau Tanja für ihre selbstlose Unterstützung und meinem Freund Benjamin für die sprachliche Korrektur des englischen Textes Dank schenken.