universität wien

Diplomarbeit

Titel der Diplomarbeit

Characterization of CD94/NKG2 receptors on cytotoxic lymphocytes

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 29.09.2009

It is an important and popular fact that things are not always what they seem. For instance, on the planet Earth, man had always assumed that he was more intelligent than dolphins because he had achieved so much - the wheel, New York, wars and so on - while all the dolphins had ever done was muck about in the water having a good time. But conversely, the dolphins had always believed that they were far more intelligent than man - for precisely the same reason.

Duglas Adams, *The Hitchhiker's Guide to the Galaxy*

Danksagung

Mein besonderer Dank gilt Prof. Erhard Hofer für die Möglichkeit, an einem so faszinierenden Thema arbeiten zu können sowie für die Betreuung dieser Arbeit und die großzügige Unterstützung auch abseits davon.

Des weiteren möchte ich meinen Kollegen Dr. Tomasz Bobrzynski, Viktoria Breinsberger, Dr. Irene Karas, Dr. Karoline Lipnik, Dr. Mingjie Wen, Dipl.-Biol. Dorit Reiche, Dr. Susanne Sattler, Dr. Bernhard Schweighofer, Mag. Caterina Sturtzel, Mag. Julia Testori und Maria Witkowski für die gute Zusammenarbeit, fruchtbare Diskussionen, Hilfsbereitschaft und Freundschaft danken.

Zudem möchte ich Dr. Stephan Blüml, Prof. Rainer de Martin, Dr. Lamia El-Housseiny, Mag. Iris Ertl, Prof. Hartmut Hengel, Dr. Renate Hofer-Warbinek, Mag. Christoph Lemberger, Prof. Hans-Joachim Lipp, Prof. Miguel López-Botet, Mag. Eva Mercé-Maldonado, Dr. Ulrike Resch, Mag. Susanne Richter, Mag. Yvonne Schichl und Dominik Stichlberger für Hilfe und Anregungen sowie die Bereitstellung von Materialien danken.

Vielen Dank an alle Freundinnen und Freunde, die mir immer den nötigen Ausgleich zum Studium geboten haben. Es war schön zu erfahren, dass ihr auch in schwierigen Zeiten für mich da seit!

Mein größter Dank gilt allerdings meiner Familie, die mir stets den nötigen Rückhalt geboten hat, um meine Zukunftspläne verwirklichen zu können und mir dabei immer größtmögliches Vertrauen und Unterstützung entgegenbrachte.

Contents

Summary

Lectin-like NK cell receptors are expressed on NK cells and a fraction of T lymphocytes and are involved in providing protection against invading pathogens and cancer. Among these, the heterodimeric NKG2/CD94 receptors, comprising activating and inhibitory family members, recognize the "self" marker HLA-E and have been implicated as receptors of major importance. The goal of the first part of this work was to investigate the distribution of the CD94/NKG2A and CD94/NKG2C receptors within peripheral blood lymphocytes (PBL) to define subpopulations of NK and T lymphocytes expressing these receptors. Flow cytometry analysis displayed subpopulations of variable sizes between different individuals. Usually expression of the inhibitory CD94/NKG2A defined the largest NK subpopulation, whereas NK cells with the activating CD94/NKG2C constituted a smaller subpopulation. Cells with expression of both receptors were also found at very low percentage. The receptors were further detected on T lymphocytes with similar distribution of NKG2A and NKG2C subpopulations. In the next part, we asked the question wether the NKG2 isoforms would provide different functions to the subpopulations. The expansion of these subpopulations in co-cultures of peripheral blood mononuclear cells (PBMC) with an HLA class Ia-negative, but HLA-E transfected cell line, was investigated as well as the degranulation upon target cell encounter as measured by CD107a surface expression. In co-culture with the HLA-E cells, the $NKG2C^+$ subset displayed preferential expansion as compared to $NKG2A^+$ cells. Major functional differences of NKG2A and NKG2C subpopulations were in freshly isolated cells an inhibition of degranulation of the NKG2A cells by HLA-E, whereas after co-culture the NKG2C cells acquired increased killing capacity for the HLA-E cells suggesting that the self-ligand HLA-E can function as triggering ligand after preactivation of the cells. Finally, the tools to investigate cellular expansion in the context of cytomegalovirus (CMV) infections of fibroblasts and endothelial cells were prepared and initially tested as NKG2 receptors have been implicated in CMV immunosurveillance. Corresponding amplifications of NK and T cell subpopulations with NKG2 receptors were observed, however were to a large degree dependent on the individual donors.

In summary, this work has defined subpopulations of NK as well as T cells by expression of NKG2 receptors for which differences in function could be shown in the context of cells with expression of the cognate ligand HLA-E and CMV infection.

Zusammenfassung

Lektin-ähnliche Natürliche Killer (NK) -Zell Rezeptoren werden von NK und einem Teil der T Zellen exprimiert und vermitteln Schutz vor Pathogenen und Tumoren. Dazu gehört die Familie der heterodimeren CD94/NKG2 Rezeptoren. Diese besitzen entweder inhibierende oder aktivierende Funktion, erkennen das “selbst-Antigen” HLA-E und spielen eine wichtige Rolle bei der Regulierung der Zellaktivität.

Ein erstes Ziel dieser Arbeit war es, die Verteilung von CD94/NKG2A und CD94/NKG2C Rezeptoren innerhalb der Lymphozyten des periphären Blutes (PBL) zu untersuchen und dadurch Subpopulationen von NK- und T- Zellen zu identifizieren. Die durchflusszytometrische Analyse ergab Subpopulationen von variierender Größe in Abhängigkeit vom individuellen Spender. Die größte Subpopulation wurde über den inhibierenden Rezeptor CD94/NKG2A definiert, wohingegen CD94/NKG2C stets von einer kleineren Population exprimiert wurde. Ein sehr geringer Prozentsatz an Zellen koexprimierte beide Rezeptoren. Dies gilt sowohl für NK- als auch T- Zellen.

Des Weiteren haben wir untersucht, inwieweit die Expression von NKG2 Isoformen eine Auswirkung auf die Funktionen der Subpopulationen hat. Dafür wurde die Expansion dieser Subpopulationen in Kokulturen von mononukleären Zellen des periphären Blutes (PBMC) mit einer HLA Klasse Ia negativen, aber HLA-E transfektierten Zelllinie analysiert. Weiters wurde die Degranulierungs- Aktivität über CD107a gemessen. In der Kokultur zeigten $NKG2C^+$ Zellen verstärkte Expansion im Vergleich zu $NKG2A^+$ Zellen. Die wichtigsten funktionellen Unterschiede zwischen NKG2A und NKG2C positiven Subpopulationen waren eine Inhibition der Degranulation von frisch isolierten $NKG2A^+$ Zellen in Abhängigkeit von HLA-E und ein HLA-E-vermittelte Aktivierung von zuvor kokultivierten, $NKG2C^+$ Zellen. Dies deutet darauf hin, dass der “selbst-Ligand” HLA-E voraktivierte Zellen über NKG2C induzieren kann.

Schließlich wurden Methoden etabliert, um Zellexpansion im Zusammenhang mit Zytomegalovirus-infizierten Fibroblasten und Endothelzellen zu untersuchen, da NKG2 Rezeptoren für die Kontrolle von CMV durch das Immunsystem eine wichtige Rolle spielen dürften. Eine vom Spender abhängige, CMV assoziierte Amplifizierung von NKG2 exprimierenden NK und T-Zell Subpopulationen wurden festgestellt.

Zusammenfassend konnten durch die in dieser Arbeit durchgeführten Experimente Subpopulationen auf NK- und T- Zellen anhand der Expression von NKG2 Rezeptoren definiert und funktionale Unterschiede im Zusammenhang mit HLA-E und CMV festgestellt werden.

1. Introduction

1.1 Reflecting "self": The major histocompatibility complex (MHC) class I

Major histocompatibility complex class I proteins are expressed, with few exceptions, by all healthy nucleated cells. The expression level varies between different cell types. For example, trophoblast and germinal cells express no MHC class I molecules and cells of the nervous system express little or none whereas lymphoid tissues express high levels of MHC class I. In humans, the corresponding proteins are termed human leukocyte antigens (HLA). Their functions are diverse ranging from antigen presentation and indication of "self" and "non-self" to protecting the foetus from rejection (Agrowal & Kishore, 2000).
MHC class I heavy chains assemble in the endoplasmatic reticulum (ER) to a heterodimer with β_2-microglobulin (β_2m). Endogenous proteins that are degraded by the proteosome and transported to the ER via TAP (transporter associated with antigen processing) are processed and loaded onto the heterodimers by the peptide loading complex. Exogenous proteins that are partially processed in endosomes and reach the cytosol can also be loaded onto MHC class I molecules. MHC-I/β_2m/peptide complexes enter the golgi network and finally reach the cell surface where they are recognized by T and NK cells (Rodgers & Cook, 2005). An important mechanism to recognize intracellular pathogens such as viruses is the presentation of pathogen-derived peptides in the context of MHC class I to cells of the immune system, in particular cytotoxic T cells (Hansen & Bouvier, 2009).
The classical MHC class I alleles (MHC class Ia) coding for HLA-A, -B and -C are highly polymorphic in the regions for the peptide-binding sites. Two or three "anchor residues" are responsible for specific peptide binding, which enables loading of diverse eight to ten amino acids long peptides.

In contrast, few alleles exist for the non-classical MHC class I (MHC class Ib) genes HLA-E, -F, and -G. HLA-G and -F diverged from MHC class Ia 5–20 million years ago. HLA-G was shown to present peptides in a similar way as MHC class Ia molecules do, and the same is probably true for HLA-F. Their expression is limited to some tissues and cell types where the functions are not yet fully clarified but contribution to immunoregulation, allergy, autoimmunity and embryonic development was implicated (Rodgers & Cook, 2005).
HLA-E arose more than 65 million years ago from MHC class Ia and is expressed in most tissues including the placenta. Rodents have several MHC molecules of this type, fulfilling various functions, whereas in humans HLA-E represents the only member (Rodgers & Cook, 2005). HLA-E is the least polymorphic of all MHC class I molecules with probably only 3 different alleles existing, two of which (HLA-E*0101 or HLA-E^R and *0103 or -E^G) are nonsynonymous resulting in a single amino acid (arginine (R) or glycine (G)) difference not located

within the peptide binding groove (Grimsley, et al., 2002; Strong, et al., 2003). When HLA proteins are co-translationally translocated to the ER, the signal peptide is cut off and either the N-terminal part or the total signal peptide is released to the cytoplasm where the proteosome further digests it and, after re-import into the ER, these peptides are loaded onto HLA-E (Bai, et al., 2000; Rodgers & Cook, 2005). Five anchor residues in HLA-E cause tight restriction on the variety of bound peptides. Classical MHC class I and HLA-G derived molecules can be loaded onto HLA-E leading to its surface expression (Sullivan, et al., 2008). Thus, examining HLA-E surface content represents an additional control mechanism how cells can monitor MHC class I expression. CD94/NKG2 NK receptors and occasionally the T cell receptor of αβ $CD8^+$ T cells can bind HLA-E supporting a primary importance for NK receptors but also a role for acquired immunity (Sullivan, et al., 2008).

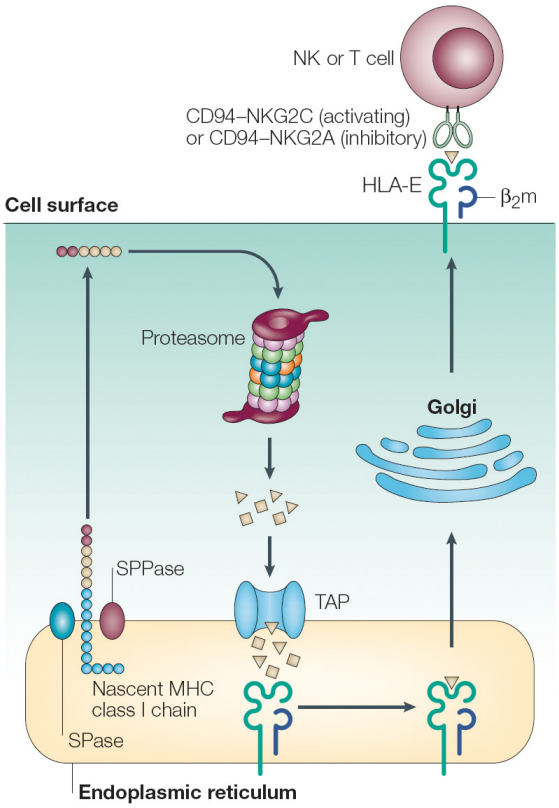

Figure 1.1: HLA-E surface mobilization and receptor engagement
Signal peptidase (SPase) and signal peptide peptidase (SPPase) mediate the release of the MHC class I leader peptide to the cytoplasm, where it is processed by the proteasome leading to short oligomeres that are re-imported to the ER in a TAP (transporter associated with antigen processing) dependent manner. β_2-microglobulin (β_2m) associated, peptide-loaded HLA-E molecules pass through the golgi network and are transported to the cell surface where they are recognized by CD94/NKG2 receptors on NK or T cells.
(Figure adapted and reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (Rodgers & Cook, 2005), copyright (2005))

1.2 Natural Killer (NK) Cells

1.2.1 The classification of NK cells within the immune system

According to the time frame, specificity of an immune reaction and evolutionary history, the vertebrate immune system consists of two functionally distinct branches:

The innate immune system includes physical and chemical barriers (skin, antimicrobial chemicals), blood proteins (the complement system) and the cellular components neutrophils, macrophages, NK cells and dendritic cells. The limited diversity of their germline-encoded receptors restrict cells of the innate immunity to the recognition of structures shared by groups of related microbes but allows a defence reaction within hours. Dendritic cells process and present antigens to cells of the adaptive immune system, serving as a link (Abbas, et al., 2007).

The evolutionary younger adaptive immune system with the cardinal features specificity, diversity, memory, tolerance and self-limitation mediates its effector functions via B lymphocytes secreting antibodies (humoral response), helper T lymphocytes activating macrophages or B cells, and cytotoxic T-lymphocytes (cellular response). Several days are needed to select and amplify antigen-specific cells or antibodies recognizing an unknown pathogen. Upon re-encounter, memory allows a highly specific, rapid response (Abbas, et al., 2007).
In 1975 it was discovered by Kiessling et. al, that a subset of mouse peripheral blood lymphocytes (PBL) kill certain tumor target cells without prior sensitization. Germline encoded cell surface receptors that do not require somatic recombination and the property of "spontaneous killing" led to the term "natural" killer cells and their classification to the innate immune system. Unlike the phagocytic cells of the innate immunity (neutrophils, macrophages) which rely solely on conserved pattern-recognition receptors, it turned out that NK cells are focused on recognition of major histocompatibility complex (MHC) molecules and belong to the lymphocytes where they make up about 15% in humans (Lanier, 2005; Wilk, et al., 2008). Ten years after their first definition the "missing self" hypothesis was proposed: Polymorphic MHC class I molecules, which are abundantly expressed on healthy cells and present self-peptides to T lymphocytes are recognized also by NK cells to distinguish between "self" and "non-self" (Ljunggren & Klärre, 1985). Tumor or virus transformed cells frequently downregulate MHC class I expression making them targets for killing.
Together with B and T cells, NK cells share a common lymphocytic progenitor and, according to their current definition, are not found in species lower than fish. Nowadays, NK cells appear to be a transitional cell type connecting innate and adaptive immune systems and having coevolved with T cells rather than being an ancient immune effector cell type (Lanier, 2005; Joncker & Raulet, 2008).

1.2.2 NK cell education and activation

NK cells use highly cytotoxic preformed lytic granules for target cell lysis. To avoid possible unspecific killing, a balance of inhibitory and activating signals controls the granule release.

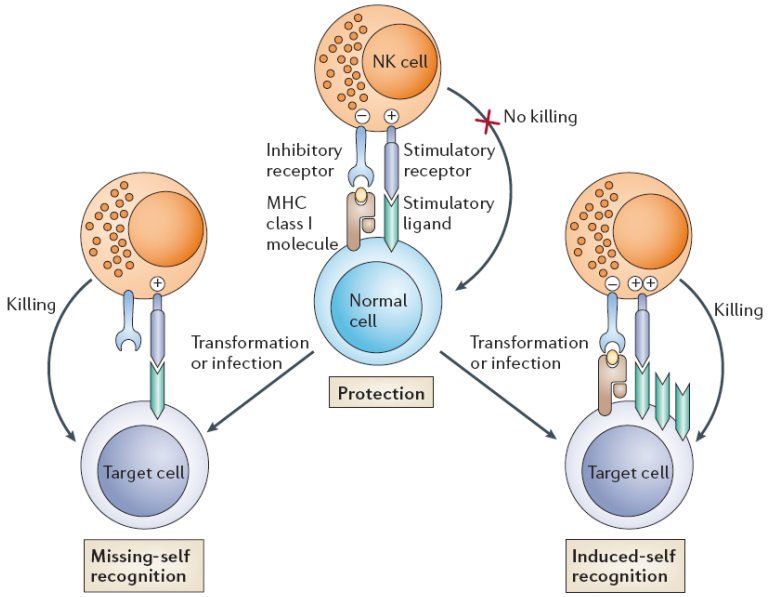

Figure 1.2: MHC class I dependent mechanisms of NK cell activation
If an NK cell encounters a normal cell, the balance between activating and inhibitory signals (mainly from MHC class I specific inhibitory receptors) provides the basis for non-reactivity to "self". Downregulation of MHC class I (missing-self) or upregulation of stimulatory ligands (induced-self) due to transformation or infection leads to NK cell activation and target cell killing. (Raulet & Vance, 2006)

If a normal cell gets encountered, inhibitory signals, coming mainly from inhibitory receptors recognizing MHC class I molecules, dominate and no activation takes place. If either stimulatory ligands for activating receptors are upregulated (induced-self recognition) or inhibitory molecules are downregulated (missing-self recognition), killer-cell activation leads to cytokine and granule release resulting in elimination of the target cell (Raulet & Vence, 2006).
To achieve self-tolerance and optimal reactivity it was proposed that NK cells have to express at least one inhibitory receptor, recognizing self-MHC class I, to get fully functional. Such cells are called "licensed" or "higher-functioning" as opposed to "unlicensed" or "hyporesponsive" (Raulet & Vence, 2006). Early models for the process of licensing focussed on the receptor acquisition during development: The "sequential-cumulative model" proposes that during a certain time frame of NK cell development, receptors are expressed stochastic but stable. Accumulation of new receptors continues until an inhibitory receptor engaged self-MHC class I with sufficient strength (Hanke, et al., 2001). Alternatively, stochastic receptor expression of a preformed initial receptor repertoire takes place and a selection process favours NK cells with "at least one" self-MHC specific inhibitory receptor. Both mechanisms were later found to be

incapable to ensure that “at least one” inhibitory receptor for “self” is expressed on each NK cell (Joncker & Raulet, 2008). The process, that NK cells are proposed to have to undergo for establishing responsiveness, was named NK cell “licensing”, “education” or “arming” and the corresponding models “arming model” or “licensing model” according to which the interaction of inhibitory receptors with MHC-class I receptor expressing cells induces responsiveness (Kim, et al., 2005; Yawata, et al., 2008). The arming/licensing models require that NK cells (and accordingly their activating receptors) are by default in an “inactive”, “unresponsive” state, for which there is no proof yet (Raluet & Vence, 2006; Held, 2008). If this is not the case, the primary attribute for a “selection” remains that NK cells have to ensure the property of tolerance for “self” (Held, 2008). An alternative viewpoint for example is taken to account in the “disarming” model where NK cells are highly functional by default and if excessive activating signaling occurs due to lack of inhibitory receptor signaling, cells do not get fully responsive and potentially acquire functional anergy (Joncker & Raulet, 2008).

Models relying solely on MHC class I inhibitory receptor expression fail to explain some aspects of NK-cell biology: Humans and mice that lack proper MHC class I expression due to mutations effecting TAP or β2m as well as mice carrying both defects resulting in undetectable MHC class I surface expression, harbour usual numbers of NK cells with a widely normal receptor repertoire and are found self-tolerant. Additionally, a substantial subset of self-tolerant cells without MHC class I inhibitory (KIR^- $NKG2A^-$) receptors exist in healthy humans and mice (Kumar & McNerney, 2005; Anfossi, et al., 2006; Joncker & Raulet, 2008). Whereas the role of MHC class I dependent “self” recognition for NK cells to become fully functional is beyond question, there is further considerable evidence that MHC class I independent mechanisms are also involved.

Recent findings indicate that “education” is quantitative and probably also reversible. In the “rheostat” model (Fig. 1.3), the quantity of inhibitory and stimulatory interactions to which cells are exposed during development provides the basis for non-reactivity to self. An equal amount of activating and inhibitory interactions results in high responsiveness, whereas an access of activating signals would lead to a reduction of responsiveness. In terms of cell activation it is still the balance of activating and inhibitory signals governing the outcome but according to this theory, the set-point can be changed if persistent conditions change, leading to tolerance of pathogens or cancer because newly arising NK cells are “used” to the altered steady-state situation (Joncker & Raulet, 2008; Brodin & Höglund, 2008).

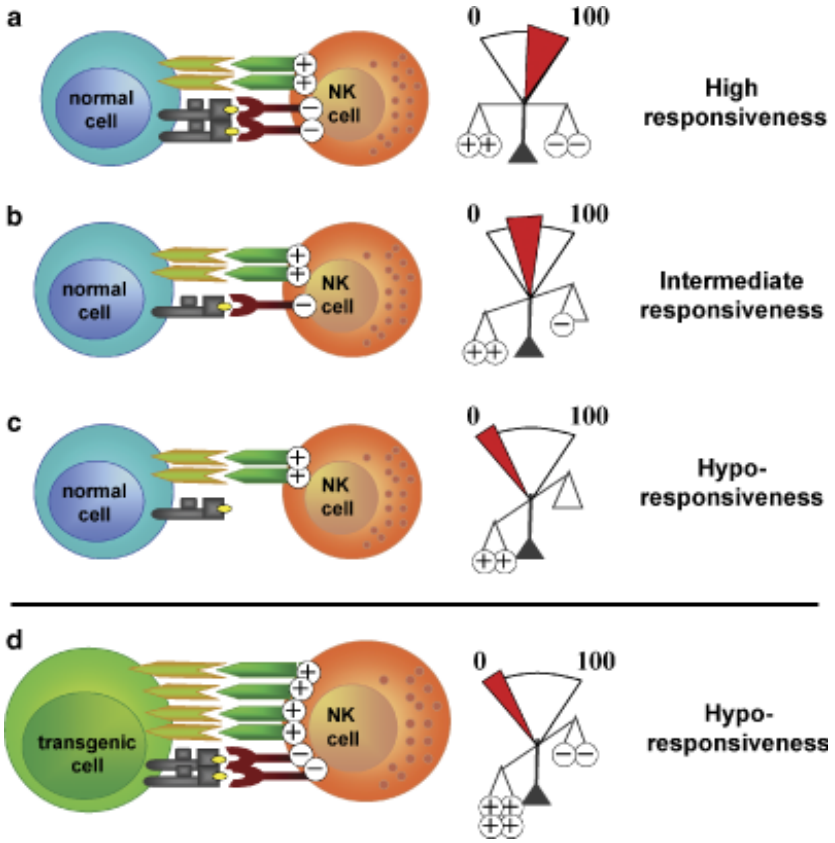

Figure 1.3: The "reostat" model of NK cell responsiveness
The amount of inhibitory and activating signals received during NK cell development governs the responsiveness. An equal amount of inhibitory and activating signals (a) results in "high responsiveness" whereas both weak or missing inhibitory "self" signals (b, c) and an excess of activating signals (d) leads to reduced reactivity (Joncker & Raulet, 2008).

Upon encounter of a target cell through adhesion molecules like LFA-1 (a β_2-integrin) binding to ICAM-1, the decision between activation and inhibition takes place in the immunological synapse. In order to avoid cell activation, signal transduction of inhibitory signals and inhibition of activating responses is necessary. It is currently thought that this is enabled by close proximity of the receptors and association with Src kinases which phosphorylate immune tyrosine-based inhibitory motifs (ITIMs). Upon phosphorylation, enzymes like SHP-1 tyrosine phosphatase are recruited which dephosphorylate immune tyrosine-based activating motifs (ITAMs) present on the adaptor proteins of many activating receptors. Furthermore, inhibition includes prevention of the actin-dependent recruitment of activating receptors to lipid rafts by SHP-1 which inactivates Vav1 dependent Rac1 activation and thereby prevents actin polymerization (Fig. 1.4). Polarization and the localized cell inhibition renders NK cells susceptible for activation taking place in different membrane areas. Induction of cytotoxicity and cytokine production in resting NK cells often requires synergy of several receptors. Much more activating than inhibitory receptors are expressed on NK cells and if activation signals exceed the inhibitory, effector functions are mediated (Watzl & Long, 2003; Barber, et al., 2004; Bryceson, et al., 2006; Long, 2008; Biassiani, 2009; Cheent & Khakoo, 2009).

Another important aspect for NK cell activation, function, proliferation and survival is the influence of cytokines. Cytokines like IL-2, IL-12, IL-15 and IL-18 as well as type I interferons (IFN-α, -β) released from various cell types have a major impact on natural killer cells and are part of the cross-talk with other cells during an immune reaction. Especially dendritic cells were shown to provide additional signals required for fully activated natural killer cells (See chapter 1.1.4). (Strowig, et. al., 2008)

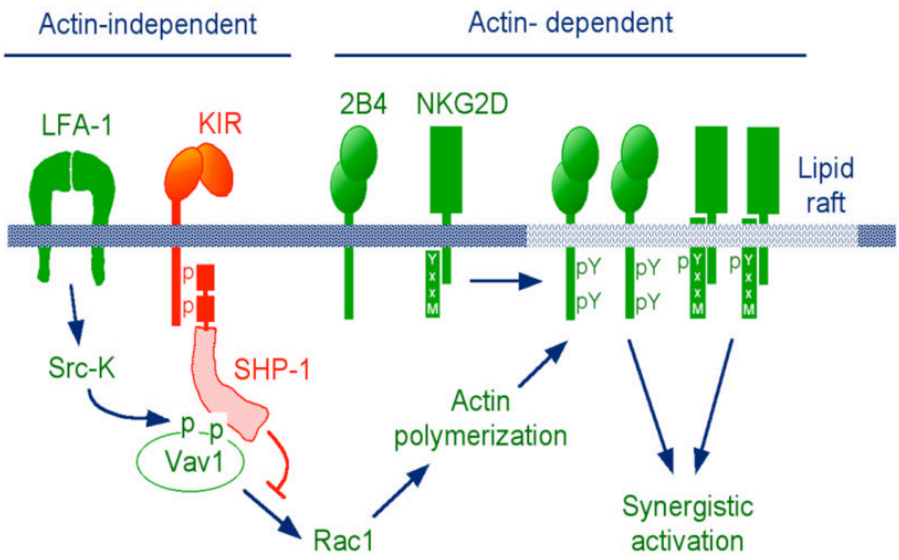

Figure 1.4: Regulation of inhibitory and activating NK cell signaling
Adhesion molecules like LFA-1 are important for the binding to a potential target cell and to induce actin polymerization required for the recruitment of activating receptors to lipid rafts and therefore major players during cell activation. If inhibitory NK cell receptors (like inhibitory KIR and CD94/NKG2A) encounter a cognate ligand, SHP-1 is recruited which dephosphorylates Vav1 and thereby prevents the induction of an activating signal (Long, 2008).

1.2.3 Receptors, adaptor proteins and ligands of human NK cells

Germline restriction necessitates NK cells to express a multitude of activating and inhibitory receptors to enable stringently controlled diverse functions. The specificity of their extracellular parts differs to a large extent but all inhibitory receptors share the conserved cytoplasmic sequence of an ITIM and adaptor proteins like Dap12 which contain ITAMs or other signaling motifs are used to transduce activating signals (Biassiani, 2009). This chapter does not exhaust but focuses on the receptors of capital importance for NK cells.

1.2.3.1 MHC class I specific receptors

The killer-cell immunoglobulin (Ig)-like receptor (KIR, formerly termed killer cell inhibitory receptor (www.ebi.ac.uk/ipd/kir, june 2009) family contains inhibitory as well as activating transmembrane receptors which are encoded by at least 15 different genes and recognize the polymorphic α1-α2 area of HLA allotypes. Recombination and gene shuffling led to a rapid

evolution and large variability of gene content and allelic polymorphism between species and individuals (Gendzekhadze, et al., 2006; Biassoni, 2009). Acquisition of KIR expression by NK cells is random and sequential resulting in stochastic expression independent of self MHC class I leading to cells co-expressing multiple KIR (Andersson, et. al, 2009; Cooley & Miller, 2009). Inhibitory KIR use ITIMs on their intracellular part to transduce signals. Activating KIR dimerize with Dap12 or FcεRIγ which contain ITAMs (Biassiani, 2009). KIR can also be expressed by T cells where they act as co-receptors and influence lymphokine production (D'Andrea, et al., 1996).

Inhibitory and activating members of the C-type lectin-like family of receptors recognize the non-classical MHC class I molecule HLA-E. In chapter 1.3 these receptors will be described in more detail.

Leukocyte Ig-like receptors (LILR) also known as Ig-like transcripts (ILTs) recognize the highly conserved α3 domain of MHC class I complexed with β_2-microglobulin resulting in broad specificity. The 14 inhibitory or activating LILR known are predominantly expressed on myelomonocytic cells. For NK cells LILRB1 coding for CD85j (LIR1/ILT2) is of special interest. It recognizes several classical and nonclassical MHC class I molecules as well as the cytomegalovirus encoded MHC class I homologue UL18 leading to inhibitory signals. CD85j was shown to interfere with NK adhesion to target cells and to inhibit CD16 dependent lysis. On the other hand, $CD85j^+$ NK cells potentially control HIV-1 replication in dendritic cells (Brown, et al., 2004; Cosman, et al., 1997; Scott-Algara, et al., 2008).

1.2.3.2 Receptors not specific for MHC class I

In contrast to MHC class I specific receptors, the majority of the receptors not recognizing MHC class I are present on the surface of most NK cells. The natural cytotoxicity receptors NKp30 (NCR3), NKp44 (NCR1), NKp46 (NCR2) which are members of the Ig-like superfamily and the lectin-like receptor NKG2D (KLRK1) are the main receptors for NK-mediated killing (Biassiani, 2009; Cheent & Khakoo, 2009).

NKp30 and NKp46 are expressed by the majority of resting and activated NK cells, whereas NKp44 is expressed upon activation (Biassiani, 2009). Not much is yet understood about the ligands of NCR but it seems that functional cooperation takes place as the engagement of any NCR results in activation of a common set of tyrosine kinases and the induction of downstream signaling of other NCR (Augugliaro, et al., 2003; Bottino, et al., 2005). NKp46 and NKp44 were shown to recognize hemagglutinin on virus infected cells (Mandelboim, et al., 2001; Arnon, et al., 2001). NKp46 is further involved in killing of *Mycobacterium tuberculosis* infected monocytes via vimentin binding (Garg, et al., 2006) and NKp44 has been suggested to

bind directly to several bacteria (Esin, et al., 2008). In addition, inhibition of NCR by specific antibodies revealed their major role in NK cell mediated lysis of tumors (Bottino, et al., 2005) and it is debated if membrane-associated heparan sulfate proteoglycans (HSPG) could serve as cellular ligands at least for NKp44 and NKp46 (Biassiani, 2009). HLA-B associated transcript 3 (BAT3) and B7 protein family member B7-H6 were identified as tumor antigens for NKp30 (Pogge von Strandmann, et al., 2005; Brandt, et al., 2009).

Like other natural killer group 2 (NKG2) proteins, NKG2D is a member of the C-type lectin-like molecules. The type II transmembrane anchored protein is expressed as a disulfide-linked homodimer and not as a heterodimer with CD94 like other NKG2 family members. NKG2D recognizges stress induced "self-proteins" expressed upon DNA damage, infection or as a result of toll-like receptor (TLR) signalling and is therefore important for tumor immunosurveillance and pathogen defence. The identification of at least 7 different ligands gives NKG2D an exceptional position among NK cell activating receptors: MHC class I related chain A (MICA), MICB, cytomegalovirus UL16-binding protein 1 (ULBP1), ULBP2, ULBP3, ULBP4 and retinoic acid early transcript 1G (RAET1G) and RAET1E are recognized. All of these ligands share the common feature of MHC-class-I-related $\alpha 1 \alpha 2$ domains but aside of that are highly variable in amino-acid sequence and domain structure. Upon ligand binding, a tyrosine motif on the associated adaptor protein Dap10 (which lacks ITAM) is phosphorylated and phosphatidylinositol-3 kinase as well as Grb2 and the effector molecule Vav1 has to interact in order to induce activation (Upshaw, et al., 2006; Eagle & Trowsdale, 2007; Bryceson & Ljunggren, 2008; Jonjic´, et al., 2008).

CD16 (FcγRIII), also termed low-affinity Fcγ receptor, is expressed by most NK cells and recognizes the Fc part of IgG. Association with the ITAM bearing CD3ζ or Fcε receptor I (FcεRI) γ chains is required for signal transduction. Antibody-coated target cells are recognized, inducing antibody-dependent cellular cytotoxicity (ADCC). CD16 engagement appears to be the only example for an activating NK cell receptor sufficient to induce degranulation in resting cells without co-activation of other activating receptors (Bryceson, et al., 2006). Aside of its important role in ADCC, Mandelboim et al. showed evidence for an antibody-independent function for lysis of some virus infected and tumor cells by recognition of a ligand other than Fcγ (Mandelboim, et al., 1999; Moretta, et al., 2008).

Signaling lymphocytic activation molecules (SLAM) 2B4 (CD244) NTB-A (SLAMF6, Ly108) and CS1 (SLAMF7, CRACC) belong to CD2 superfamily and are thought to act as co-receptors during activation of natural cytotoxicity. The proteins have in common that they show homophilic interactions or recognition of proteins encoded within the CD2 gene family. All of them contain an extracellular N-terminal nondisulfide bonded V type Ig-like domain and a membrane-proximal C2 domain. SLAMs play an important role in innate and adaptive im-

munity. Immunoreceptor tyrosine-based switch motifs (ITSM) on their cytoplasmic tail can, similar to ITIM, recruit Src homology 2 (SH2) domain-containing proteins. Receptor type and cellular context influences wether an ITSM transduces an activating or an inhibiting signal. In healthy individuals, SLAMs are activating NK cell receptors (Ostrakhovitch & Li, 2006; Biassoni, 2008).

NKR-P1A (CD161) is a C-type lectin receptor expressed on a subset of NK and 25% of peripheral T cells (Lanier, 1994; Lanier, 2005). Lectin-like transcript 1 (LLT1 or CLEC2D) is a ligand for NKR-P1A and interaction inhibits NK and $CD8^+$ T cells but fails to inhibit $CD4^+$ T cell activation. LLT1 is expressed on NK cells, T cells, activated dendritic cells and B-cells suggesting a function in the cross-talk of NK and antigen-presenting cells (APC). The exact nature of signals by CD161 is still to be clarified (Aldemir, et al., 2005; Rosen, et al., 2008).

The ability to recognize conserved structures on invading microorganisms (pathogen-associated molecular patterns, PAMPs) makes Toll-like receptors (TLRs) to important players of innate immunity. NK cells express TLR 3 and 9 which recognize double-stranded RNA from viruses and non-methylated CpG DNA from bacteria, respectively. These interactions could be especially important during early recruitment to inflammatory sites (Sivori, et al., 2004; Tsan, 2006).

1.2.4 Functions of NK cells in human peripheral blood and the assignment to NK subpopulations

The exertion of spontaneous cytotoxicity originally characterized natural killer cells but recent studies have clearly shown that regulation of immune responses via production of cytokines is an equally important contribution of NK cells to an immune reaction (Strowig, et al., 2008).

The highly organized multifactor process cytotoxicity can be mediated by three different pathways that results in the induction of apoptosis in the target cell. (Chavez-Galan, et al., 2009) Membrane blebbing, chromatin margination, and the breakdown of chromosomal DNA characterize apoptotic cells and finally end up in cell death (Schwartz, et al., 1992).
Granule-dependent exocytosis is the most important NK cell effector mechanism (Lanier, 2005). Upon activation, preformed lytic granules are guided by microtubule mobilization to the immunological synapse where lytic molecules are released towards the target membrane. The exact mechanism how this pathway works is still debated but the importance of perforin, which is found soluble within granules and can form cylindrical pores in membranes, is clear. Depending on the proposed mechanism, perforin either forms pores in the target membrane, allowing lytic molecules to path through, or it enables the release of these molecules after getting endocytosed together in vesicles by the target cell. Granzymes and granulysin can serve as lytic molecules that, once inside the cell, induce apoptosis pathways (Chavez-Galan, et al., 2009).

Expression of death receptor ligands like TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) and their interaction with TRAIL-R1 or -R2 or Fas, respectively, on the target cell surface is the second pathway leading to apoptosis. FasL can be expressed as membrane-anchored trimer or be stored in intracellular microvesicles and expressed on the cell surface upon stimulation. Besides, membrane shaping results in soluble FasL which can be either pro- or anti-apoptotic, depending on the mode of interaction either as a monomer (anti-apoptotic) or as a multimeric structure and if aggregated with extracellular matrix proteins (pro-apoptotic). At last, death receptor signaling leads, depending on the cell type, to active caspase-8 or release of pro-apoptotic factors from the mitochondria ending up in cell death (Chavez-Galan, et al., 2009).
Last, the cytokine tumor necrosis factor can, aside of other functions, induce apoptosis via crosslinking of TNF with TNF receptor type 1, which carries cytoplasmic death domains (Chavez-Galan, et al., 2009).

Cytokines released by NK cells participate in immunomodulation of innate and adaptive immunity and fulfill direct antiviral functions (Strowig, et al., 2008). Production of tumor necrosis factor (TNF) and the type I interferons IFN-α/β and IFN-γ suppresses viral replication, enhances NK cell mediated cytotoxicity and exerts regulatory functions on the immune reaction (Biron & Brossay, 2001). The effects of IFN-γ are manifold: It upregulates cell-surface MHC class I, increasing the potential to recognize foreign pathogens and induced changes in the composition of the proteasome are thought to positively influence quantity, quality, and repertoire of peptides for class I MHC loading. Decreasing the local blood flow rate and upregulation of chemokine and adhesion-molecule expression by IFN-γ recruits cells of the immune system. Moreover, microbicidal effector functions of macrophages are activated and isotype-switching of B-cells is promoted (Schroder et al., 2004).

The cross-talk of NK cells with dendritic cells (DC) is of particular importance. On the one hand, NK cells are activated and induced to proliferate by mature DC via IL-2, IL-12, IL-15, IL-18 and INF-α/β, on the other hand, maturation of DC is triggered by NK cells via TNF and IFN-γ. Interestingly, immature DC are susceptible to NK mediated killing because of low-level MHC class I expression, and maturation is accompanied by MHC class I upregulation which acts protective (Degli-Esposti & Smyth, 2005). Mature DC efficiently prime Th1 cells and the interplay with NK cells was shown to be able to induce $CD8^+$ T cell-mediated anti-tumor responses in the absence of $CD4^+$ T cell help. Secondary lymphoid organs are another site of NK-DC interaction. Upon inflammation, NK cells are recruited to the T cell zone in lymph nodes where close proximity with DC can activate NK cells to produce INF-γ which mediates Th1 polarization required to prime an immune response (Gregoire, et al., 2007; Strowig, et al., 2008). Another level of complexity was added by the finding that NK cells can also induce differentiation of monocytes into DC via direct contact, GM-CSF and CD40 ligand (CD154)

release (Zhang, et al., 2007).
To tightly regulate an immune reaction, other cells can also impair NK cell functions for example by secretion of IL-10 produced by T cells, macrophages and DC (Couper, et al., 2008) and TGF-β released by regulatory T cells or tumors cells (Wahl, et al., 2006).

The quantity of neural cell adhesion molecule (NCAM, CD56, Leu-19, NKH-1) expression on natural killer cells distinguishes two functionally distinct subsets with the major functions "cytokine production" or "cytotoxicity". (Lanier, et al., 1986) CD56 is expressed by virtually all NK cells, even so, its function has yet to be defined. Cells expressing high levels of CD56 on their cell surface are referred to as $CD56^{bright}$ and those expressing 5 to 10 fold less as $CD56^{dim}$ NK cells. Both subsets express the activating receptors NKp30, NKp46 and NKG2D but their gene expression profiles vary to a large extent with more than 470 differentially expressed transcripts, almost 2/3 of which are exclusively found in one subset (Wilk, et al., 2008) .

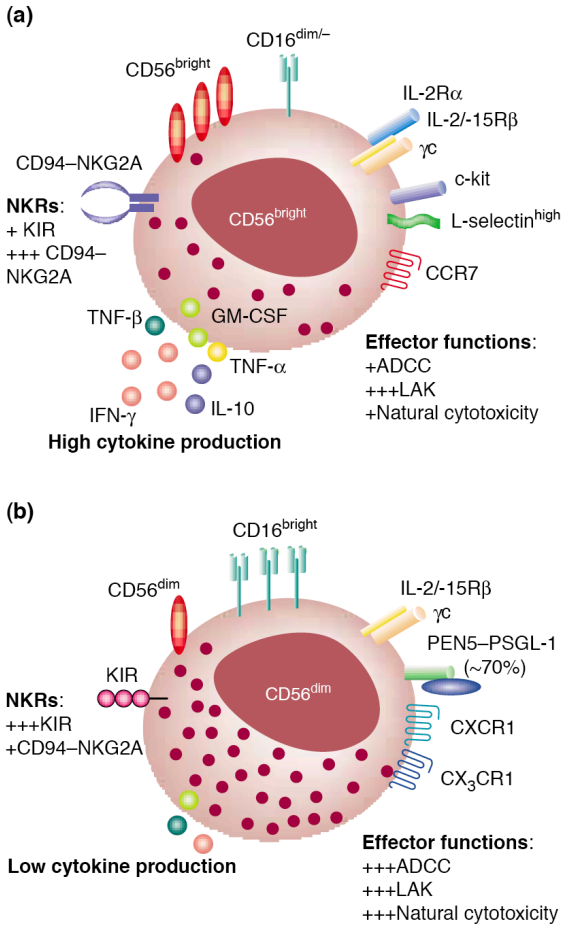

Figure 1.5: Surface protein expression & functions of NK cell subpopulations
$CD56^{bright}$ NK cells express high levels of inhibitory CD94/NKG2A, low levels of CD16, are potent cytokine producers but show weak cytotoxic activity. Conversely, $CD56^{dim}$ NK cells express high levels of CD16 (making them to potent mediators of ADCC), few CD94/NKG2A, produce low levels of cytokines but are highly cytotoxic. Both subsets are equally capable of lymphokine activated killing (LAK). (see text for further details)
KIR, killer Ig-like receptors; NKR, NK receptor; ADCC, antibody-dependent cellular cytotoxicity (Cooper, et al., 2001).

The “classical”, cytotoxic natural killer cells make up 90% in peripheral blood and are of the $CD56^{dim}$ phenotype (Wilk. et al., 2008). KIR, high surface levels of CD16 as well as intracellular perforin content and expression of certain chemokine receptors make $CD56^{dim}$ NK cells to potent effector cells. CXCR1 (CXC chemokine receptor 1) and CX_3CR1 are “site of inflammation markers” and binding of the respective ligands IL-8 (CXCL8) and fractalkine (CX_3CL1) was shown to induce migration of $CD56^{dim}$ NK cells (Campbell, et al., 2001; Berahovich, et al., 2006; Moretta, et al., 2008). Various cell types like macrophages, neutrophils and endothelial cells release these cytokines during inflammation leading to NK cell recruitment. Furthermore, precursors of dendritic cells (DC) accumulate in inflamed tissues where they become immature dendritic cells (iDC) that secrete CXCL8 and CX_3CL1 upon antigen capture. The resulting cross-talk between iDC and NK cells is likely to induce iDC development to mature antigen presenting cells which may in turn produce cytokines that recruit further NK cells. Finally the mature DC are induced to migrate to secondary lymphoid organs. (Moretta, 2002) The chemotactic agonist Chemerin, the ligand for ChemR23 which is expressed on $CD56^{dim}$ but not $CD56^{bright}$ cells, is another chemokine important for the attraction to inflamed sites. (Moretta, et al., 2008) Despite these recruitment mechanisms, in several inflammatory sites the majority of NK cells was found to be $CD56^{bright}$ (Strowig, Brilot & Münz, 2008). This subset makes up only 10% of peripheral blood NK, does not express most KIR isotypes and either no or low density of CD16 but high levels of NKG2A and the secondary lymphoid organ (SLO) homing markers chemokine (C-C motif) receptor 7 (CCR7), CD62L (L-selectin) and CXCR3. Upon stimulation with proinflammatory molecules, 56^{bright} NK are potent producers of cytokines like IFN-γ, TNF, GM-CSF, IL-10 and IL-13, depending on the stimuli. Resting $CD56^{bright}$ $CD16^{-}$ cells lack perforin expression and require prolonged activation to become cytotoxic (Carson, et al., 1997; Ferlazzo, et al., 2004; Strowig, et al., 2008, Poli, et al., 2009). The overrepresentation of $CD56^{bright}$ cells at sites of inflammation may be due to the ability to bind macrophage inflammatory protein (MIP) -1α, MIP-1β, and RANTES (CCL5) via CCR5. Another possibility is that $CD56^{dim}$ cells differentiate into $C56^{bright}$ cells upon stimulation with certain cytokines (Takahashi, et al., 2006; Loza & Perussia, 2004) although several studies suggest a differentiation of $CD56^{bright}$ into $CD56^{dim}$ upon prolonged activation. (Dalbeth, et al., 2004; Strowig, et al., 2008) In addition, DC mediated stimulation is likely to preferentially induce $CD56^{bright}$ expansion (Vitale, et al., 2004). The reciprocal expression of the receptor-ligand pair CD62L and PEN5-PSGL-1 by $CD56^{bright}$ and $CD56^{dim}$ NK cells, respectively, suggests a potential interaction of the two subpopulations (Farag & Caligiuri, 2006). In consistence with the homing marker expression, 75% of NK cells in lymph nodes are $CD56^{bright}$. Because lymph nodes harbor 40% of total lymphocytes, 5% of which are NK cells and peripheral blood contains only 2% of the total lymphocytes (10% NK), $CD56^{bright}$ natural killer cells may outnumber $CD56^{dim}$ in the human body (Ferlazzo, et al., 2004). The existence of so many potent immunoregulatory $CD56^{bright}$ cells at various sites of the immune system further emphasizes that NK cells are more than just effective killers.

1.3 The human natural killer group 2 (NKG2) receptor family

1.3.1 Genomic organization and evolution

The NKG2 proteins are encoded by the corresponding KLRC genes located within the NK gene cluster on chromosome 12 (Plougastel & Trowsdale, 1998). In humans, the family is represented by NKG2-A, -B, -C, -D, -E, -F, -H with NKG2A/B and NKG2E/H being splice variants of the same gene (Bellon, et al., 1999; Brostjan, et al., 2002). CD94 (KLRD1) and the NKG2 proteins are highly conserved during speciation and probably arose at the time of the first tetrapods >400 million years ago (Biassoni, 2009). Though being evolutionary conserved, polymorphism screening of NKG2-A and -C revealed ten polymorphisms of non-coding regions and introns and one variation in the transmembrane region of NKG2A in the Japanese population as well as homozygous deletion of NKG2C in about 4% of healthy Dutch and Japanese individuals (implying 20% carrying the deletion haplotype) (Hikami, et al., 2003; Miyashita, et al., 2004). The homodimeric NKG2D receptor has only distant functional and structural relationship to the other family members and was therefore described separately in chapter 1.2.3.1. The CD94/NKG2 protein family consists of inhibitory as well as activating receptors. Inhibitory NKG2A and activatingNKG2C and E have 56% amino acid homology in the intracellular and transmembrane domains and more than 94% homology in their extracellular domains. The extracellular similarity allows binding to the same cognate ligand HLA-E (Borrego, et al., 2006).

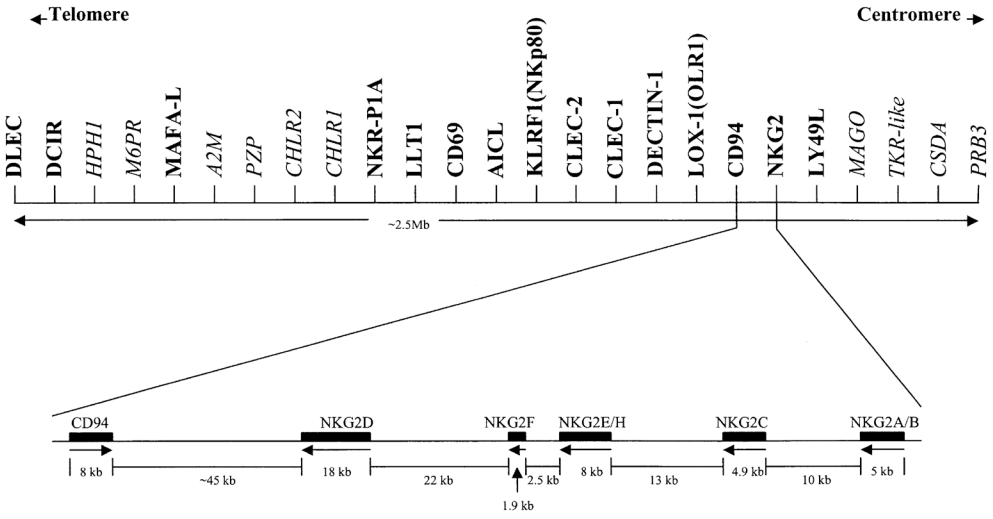

Figure 1.6 Genomic location and organization of CD94 and NKG2 genes on chromosome 12
All genes coding for NKG2 receptors as well as the gene the invariant CD94 chain (except for homodimeric NKG2D) locate in close proximity within the NK cell receptor complex on chromosome 12 (Borrego, et al., 2001).

1.3.2 CD94/NKG2 receptor structure and interaction with HLA-E

The CD94 and NKG2 receptor chains are type II transmembrane glycoproteins with an extracellular domain containing a C-type lectin-like domain (CTLD) which is connected via a stalk region to the transmembrane and cytoplasmic part (Sawicki, et al., 2001). Historically, C-type (or Ca^{2+} dependent) lectins were defined as proteins containing homologous modular carbohydrate recognition domains that bind carbohydrate moieties in a Ca^{2+}-dependent manner and contain invariant disulfide bonds (Boyington, et al., 1999). However, less than 10% of CTLD (in *Caenorhabditis elegans*) were found to contain features for Ca^{2+} binding sites and sugar recognition is also not shared by all members of the superfamily. The structural similarity of all CTLD is thought to be a consequence of evolution from a common ancestor protein (Drickamer, 1999; Sawicki, et al., 2001). The CTLD of the CD94/NKG2 receptors are subclassified to the "C-type lectin-like domains of NK receptors" (NKDs) which are Ca^{2+} independent and appear to be rather protein binding (Sawicki, et al., 2001). Except for the homodimeric NKG2D, dimerization of the NKG2 protein with CD94 via disulfide-linkage is necessary to become a functional receptor expressed on the cell surface (Carretero, et al., 1997).

Nanomers, derived from the leader peptide of different MHC-class I proteins bind to HLA-E. The amino acid composition of these nanomers and the HLA-E haplotype influence the thermal stability of the peptide/HLA-E complex, its surface expression level and the affinity of the CD94/NKG2-peptide/HLA-E interaction (Strong, et al., 2003; Hoare, et al., 2008). Although inhibitory as well as activating CD94/NKG2 receptors both recognize HLA-E/peptide complexes, the binding affinity of the inhibitory CD94/NKG2A is always found to be 6-fold higher than the interaction of the triggering CD94/NKG2C (Kaiser, et al., 2008), whereas CD94/NKG2E shows very similar binding properties to CD94/NKG2A. The HLA-E haplotype does not have an effect on these affinity differences (Kaiser, et al., 2005).

The major function of the NKG2 chain in the heterodimeric receptor might be to provide the signaling capacity. Differences in three amino-acids within the interface of CD94 and NKG2-A or -C indirectly account for the ligand affinity-variation possibly due to subtle conformational changes in the invariant CD94 protein, that contains the most important interaction sites with HLA-E and is likely to exclusively contact the peptide (Kaiser, et al., 2005; Kaiser, et al., 2008; Sullivan, et al., 2008) This kind of interaction appears to enable the maximal ability to recognize slight peptide-differences (Hoare, et al., 2008).

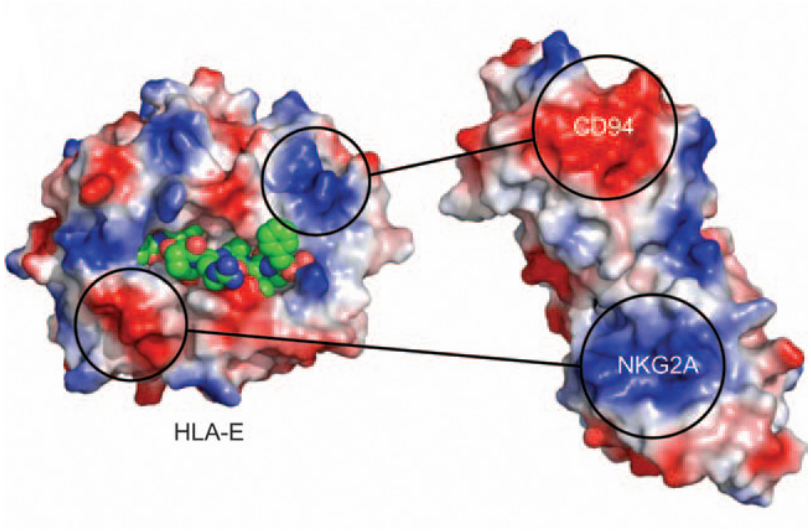

Figure 1.7 Interaction of NKG2A with the HLA-E/peptide complex
Schematic view of HLA-E/peptide and CD94/NKG2A crystal structure. Acetic regions (red) interact with basic regions (blue) with an affinity dependent on the bound peptide (green, HLA-G leader peptide is shown) (Sullivan, et al., 2008).

1.3.3 CD94/NKG2 receptor signaling and membrane dynamics

CD94/NKG2A and CD94/NKG2B comprise the inhibitory NKG2 receptors. Dimerization of CD94 or the splice variant CD94-T4 lead to functional receptors recognizing the cognate ligand HLA-E. Preferential association of CD94-T4/NKG2B and CD94/NKG2A is probably due to the absence of a stem region in both NKG2B and CD94-T4 (Lieto, et al., 2006). Two ITIM motifs on the intracellular NKG2 part facilitate, upon phosphorylation, binding of SHP-1 and SHP-2 phosphatases which suppress activating responses (Kabat, et al., 2002). Perturbing the actin cytoskeleton–dependent recruitment of activating receptors to lipid rafts, as described in chapter 1.1.2, is an important mechanism by which NKG2A and B interfere with NK cell activation. To maintain a continuous supply for interaction with HLA-E expressing cells, CD94/NKG2A receptors recycle between the cell membrane and the cytoplasm independent of ligand binding or resulting inhibitory signaling in an active cellular process that requires cytoskeletal rearrangement (Borrego, et al, 2002).

CD94/NKG2C and CD94/NKG2E/H lack ITIM motifs on their intracellular part, instead they have a charged lysine residue in the tansmembrane region that noncovalently associates with the ITAM bearing DAP12 adaptor molecule. For efficient cell surface expression, association of all three components, the CD94 and NKG2 receptor chains as well as the DAP12 molecule, is necessary (Lanier, et al., 1998; Bellon, et al., 1999). In contrast to inhibitory receptors, aggregation and activation of activating CD94/NKG2 requires an intact cytoskeleton and ATP. PYK-2 kinase, microtubule organizing center (MTOC) and paxillin are recruited to the site of target cell contact to permit an activating synapse (Borrego, et al, 2002). MAPK was also shown to be involved in the activating signaling pathway (Carretero, et al., 2000). Like CD94/NKG2A, also CD94/NKG2C is a long-lived receptor that shuffles between the cell membrane and the

cytoplasm. Protein transport inhibitor Brefeldin A treatment interferes with CD94/NKG2C but not CD94/NKG2A trafficking indicating that different mechanisms are involved (Borrego, et al., 2002).

An exception among the NKG2 protein family is NKG2F. It has a truncated extracellular domain, contains a positively charged transmembrane residue as well as an ITIM-like structure on its intracellular domain. Expression is limited to intracellular compartments which is likely due to its inability to associate with CD94. So far, the physiological role of NKG2F remains unclear (Kim, et al., 2004).

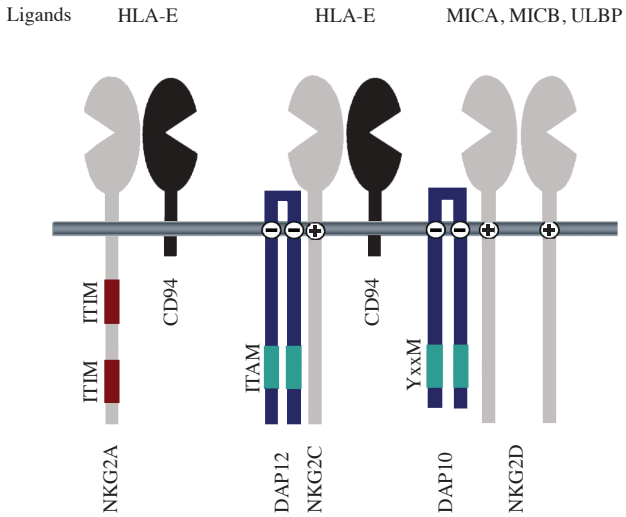

Figure 1.8 NKG2 receptors, adaptors and signaling motifs
Inhibitory NKG2A carries signaling motifs (ITIMs, immune tyrosine-based inhibitory motifs) on its intracellular part. Both activating NKG2-C and -D lack ITIMs but carry positively charged transmembrane residues which allow the association with adaptor proteins. DAP12 carries ITAM (immune tyrosine-based activating motifs) and DAP10 contains the SH2 domain binding amino acid sequence YxxM.
(Figure adapted and reprinted by permission from Macmillan Publishers Ltd: [Molecular Immunology] (Borrego, et al., 2001), copyright (2001))

1.3.4 CD94/NKG2 receptor expression and function in PBL subpopulations

We are only beginning to understand the molecular mechanisms controlling CD94/NKG2 expression. Regulation of the transcription from multiple starting sites and posttranscriptional mechanisms are suggested to play the major role and a variety of cytokines like IL-2, IL-15, IFN-α, and IL-21 was shown to be important for regulation and induction of CD94/NKG2 surface expression (Borrego, et al., 2006).

The majority of NK cells and subpopulations of T cells are the only PBL were these proteins are found, the inhibitory CD94/NKG2A constituting the predominant heterodimer. The functions exhibited by CD94/NKG2 proteins are dependent on the cell type:

Although co-activation of other receptors might, as described above, be necessary, CD94/NKG2 receptors represent largely independent, competent recognition units on natural killer cells that

transmits information about "self" and "non-self" in order to control killer-cell activation. By contrast, on T cells, CD94/NKG2 and other natural killer cell receptors (NKRs) are likely to act mainly as co-stimulatory molecules either supporting or suppressing T cell receptor mediated signaling. Differential expression of adaptor proteins and association with alternate adaptor molecules is a possible explanation for this unusual duplicity in function in innate and adaptive immunity (Snyder, et al., 2004).

Mainly $CD8^+$, $\gamma\delta TCR^+$ and D1d-restricted $CD4^-$ $CD8^-$ or $CD4^+$ $CD8^-$ T cells (NKT) but also some $CD4^+$ T cells express NK cell receptors like KIR and NKG2 (Mingari, et al., 2005; Tilburgs, et al., 2009). NKG2A expression was found to be a clonal feature of cytotoxic T-lymphocytes (CTL) and T cell receptor (TCR) antigenic specificity seams to dictate its expression (Jabri, et al., 2002). Inconsistent reports exist about the function of CD94/NKG2A on $CD8^+$ T cells: CD94/NKG2A was found to be either sufficient (Malmberg, et al., 2002; Mingari, et al., 1998) or insufficient (Romagnani, et al. 2002; Miller, et al., 2002) for inhibition of T cell receptor mediated signaling. Conflicting results exist also about CD94/NKG2 expression on $CD4^+$ T cells. Whereas some observed CD94/NKG2 only on Th1 cells, which provide cytokine-mediated "help" to cytotoxic T cells (Meyers, et al., 2002; Graham, et al., 2007), others reported expression by both Th1 and Th2 (which provide B-cell help) upon CD3-mediated activation but an impact of CD94/NKG2A stimulation on cytokine production only for Th1 (Romero, et al., 2001).

Taken together, although many open questions remain, increasing evidence suggests that inhibitory and activating CD94/NKG2 receptors play an important role not only for NK cells but also for the regulation of T cell responses to self antigens and viral infections (Pederson, et al., 2002; Mingari, et al., 2005)

1.4 Human peripheral blood T cells expressing "natural killer cell receptors"

The expression of receptors, originally found on NK cells, by T cells is dependent on the developmental stage, activation state, and genetic background of the cells. The term NKT cells is often used to refer to these cells but a clear definition is still missing which makes the usage misleading. Basically, one can distinguish between three subpopulations of T cells expressing NK cell receptors: "Conventional" T cells characterized by the expression of αβ T cell receptor (αβTCR) (1), "non-conventional" T cells harbouring either γδTCR (2) or a semi-invariant TCR comprised of an in-frame rearranged Vα24-Jα18 and a β chain (from a restricted repertoire) which makes them CD1d restricted (3). The invariant TCR of the latter precisely defines them and here they will be referred to as iNKT cells whereas (1) and (2) are named NKR^+ αβ T cells and NKR^+ γδ T cells, respectively (Matsuda, et al., 2001; Godfrey, et al., 2004; Mingari, et al., 2005; Wakao, 2009). The receptors found include amongst others LIR1 (CD85j), CD94/NKG2 and KIR (Mingari, et al., 2005; Tilburgs, et al., 2009; Kulkarni, et al., 2008) with most research dealing with expression of the inhibitory isoforms.

1.4.1 NKR$^+$ αβ T cells

NKR$^+$ αβ T cells include CD4$^+$ and CD8$^+$ cell though the majority has a CD8$^+$ phenotype. KIR$^+$ cells of both subsets were shown to accumulate with age and are enriched for effector memory cells (not expressing CCR7, CD27, and CD28) suggesting a function during effector memory cell responses (van Bergen, et al., 2004; Anfossi, et al., 2001). The same was found for LIR1$^+$ CD8$^+$ but not NKG2A$^+$CD8$^+$ (Anfossi, et al., 2001). The mechanisms governing NKR receptor expression are yet to be defined though it is clear that the factors influencing different NKR can be distinct (Vivier & Anfossi, 2004).
So far, the functional consequence of NKR on αβ T cells is unclear. For KIR, it is reported that expression may protect against activation-induced cell death of memory T cells and their expression could influence peripheral tolerance (Ugolini, et al., 2001; van Bergen, et al., 2004). Cross-linking of activating KIR on CD4$^+$ in addition to TCR signaling and absence of inhibitory KIR, activated the cells for proliferation which hints a function during initiation and for sustaining an immune response but also opens the possibility for a role in autoreactivity (Mandelboim, et al., 1996; Namekawa, et al., 2000). Inhibitory KIR or LIR1 co-ligation with TCRs on CD8$^+$ T cells was reported to impair cytoskeletal reorganization required for TCR signaling and to downregulate cytotoxicity and cytokine production (Vivier & Anfossi, 2004). CD8$^+$ T cells can express NKG2D and binding of the ligand MICA was shown to activate them (Bauer, et al., 1999). For CD94/NKG2 receptor expression and function see chapter 1.3.4.
Importantly, expression of NKR on T cells does not necessarily make them more or less efficient effector cells because the efficacy of an inhibitory NKR signaling depends on the intensity of a simultaneously occurring activating signal (Vivier & Anfossi, 2004).

1.4.2 iNKT

This subset makes up less than 0.1% of PBL in healthy human subjects and are composed of CD4$^+$, CD8$^+$ and double negative cells with implications for distinct physiological functions because of differential cytokine secretion and unequal NK-cell as well as chemokine receptor expression (Lee, et al., 2002; Godfrey, et al., 2004). The ligand for their T cell receptor, CD1d, is mainly expressed on antigen presenting cells like DC, macrophages and B cells which qualifies theses cells as potential primary interaction partners (Lee, et al., 2002). CD1 molecules are constituted, similar to MHC class I, of a heavy chain associated with β_2m light chain. All four CD1 isoforms expressed by humans present lipid antigens to T cells. CD1d has only distant relation to CD1a, b and c according to sequence homology (Vincent, et al., 2003). Self or foreign lipid antigens, loaded onto hydrophobic antigen binding clefts of CD1d are recognized in a yet poorly understood manner. However, this interaction enables recognition of antigens derived from microbes, other parasites and some types of cancer (Borg, et al., 2007; Kronenberg & Kinjo, 2009). Both “helper” and “effector” functions are carried out by the various subsets of

iNKT cells. The capacity for cytolytic activity and regulation of DC, NK and T cell activation denote their importance at the crossroad of innate and adaptive immunity even though adaptive functions are thought to dominate (Lee, et al., 2002; Vincent, et al., 2003; Minami, et al., 2005). Occasional promotion of undesirable immune responses like allergy, atherosclerosis and autoimmune diseases corroborate the importance of iNKT and signify broad clinical potential (Godfrey & Kronenberg, 2004). The NK cell receptors CD94/NKG2A and 2B4 are almost exclusively expressed by CD4 negative iNKT whereas CD161 is highly expressed by all iNKT cells (Lee, et al., 2002; Kim, et al., 2002). It was recently shown that KIR are found on iNKT at frequencies usual for T cells and that allogeneic activation of iNKT requires KIR (Patterson, et al., 2008). iNKT are bona fide αβ T cells but comprise only a very small fraction with still little knowledge about their basic function available. Most work about NKR expression on T cells distinguishes only between CD4 or CD8 expression on T cells.

1.4.3 γδ T cells

Like iNKT cells, this subset appears to locate between the innate and adaptive immune system. The γ and δ chain of the TCR are variable and so is the antigen specificity of different TCR dimers (Casetti & Martino, 2008). Most γδ T cells do not express CD4 and CD8 and are found in epithelial and lymphoid tissues and blood, where they comprise 1-5% of lymphocytes (Carding & Egan, 2002). γδ T cells can be divided in different functional subsets according to their allelic TCR composition. A wide array of antigens can be recognized due to MHC unrestricted recognition that does not require processing of the antigen (Carding & Egan, 2002). They recognize bacterial metabolites (phospho or aminoantigens), virus antigens, tumor antigens, glycolipids in the context of CD1, and stress-induced MHC class I related molecules (like MIC family proteins) and exert cytolytic activity and production of cytokines like INF-γ and T_H1 cytokines (Gober, et al, 2003; Münz, et al., 2005). A subset of γδ T cells is found to be CD1c restricted but the majority of γδ T cells in blood carries the Vγ9–Vδ2 TCR. After birth, the TCR variety progresses towards oligoclonality reaching more than 97% of cells in blood having the Vγ9 and Vδ2 rearrangements until the age of 6-10 (Spada, et al., 2000; Carding & Egan, 2002). Phosphoantigens from a wide spectrum of microorganisms were found to be recognized by this T cell subset but also other non-phosphoantigens. Similar to toll-like receptors, non-peptide antigens are scanned by the γδ TCR in order to check the environment for conserved metabolic signatures and to recognize infections and “abnormal self” (Poupot & Fournie, 2004). Very little knowledge exists about NKR on γδ T cells. MHC class I inhibitory receptor expression (KIR, but mainly NKG2A) was shown to functionally inhibit γδ T cells upon ligand binding, but expression of inhibitory NKR correlated with the potential for strong lytic activity (Halary, et al., 1997; Bakker, et al., 1998).

1.5 Human Cytomegalovirus (HCMV)

1.5.1 Classification, structure and evolution

HCMV belongs to the herpesviruses (Human herpesvirus 5 (HHV-5), genus *Cytomegalovirus*, subfamily *Betaherpesvirinae*, family *Herpesviridae*), which are among the largest and most complex of viruses (Sinclair & Sissons, 2006; Davison, 2002). The huge family of herpesviruses is found in numerous species ranging from mollusks to vertebrates and humans. Long-standing coevolution with their host leads to a narrow range of species-tropism with most of the 120 yet identified species (Davison, 2002) being associated with a single host species. Because herpesviruses evolve with their hosts they also suffer extinction with them (Davison, 2002; Tischer & Osterrieder, 2009). Virion structure, antigenic and biological properties and DNA sequence comparison was used to identify members of the *Herpesviridae* and to subclassify them into the families α-, β-, and γ-*Herpesviridae* with CMV belonging to the second (Davison, 2002).

The double stranded DNA genome of HCMV contains repetitive elements flanking two unique sequence-regions termed unique short (US) and unique long (UL). The genome exceeds 235 kb, with the potential to encode over 220 proteins, packaged in an icosahedral protein structure, the capsid, which is enveloped by a proteinaceous matrix (the tegument). A lipid bilayer envelope with numerous viral glycoproteins completes the 200 to 300 nanometer sized virion (Griffiths & Grundy, 1987; Greijer, et al., 2000; Crough & Khanna, 2009).

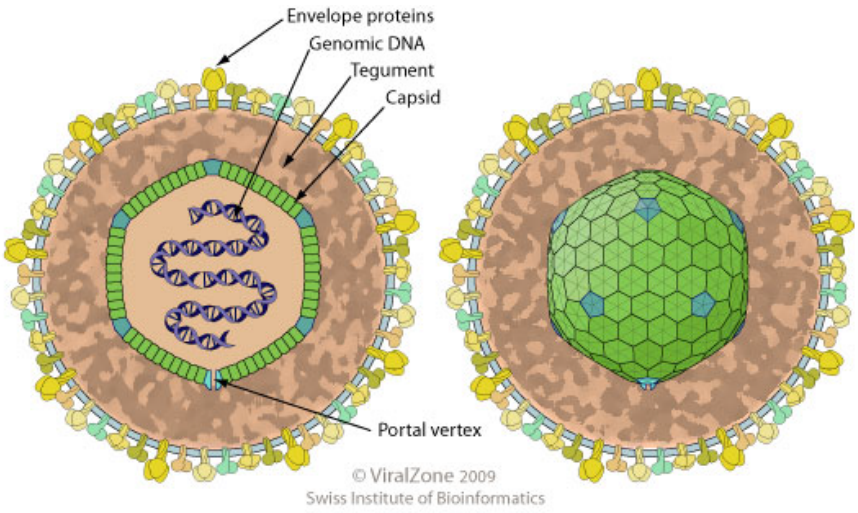

Figure 1.9 HCMV virion structure
Typical structure of herpesviruses with lipid-envelope embedded glycoproteins and an icosahedral capsid containing the viral DNA. (http://expasy.org/viralzone/all_by_protein/180.html)

1.5.2 Epidemiology

HCMV can be transmitted person-to-person via saliva, tears, urine, stool, semen, and breast milk, sexual contact, placental transfer, blood transfusion, solid-organ transplantation, or hematopoietic stem cell transplantation (Sia & Patel, 2000). De novo infection with CMV apparently occurs throughout the lifespan at a rate of around 0.5–1% per year up to the age 60–65. (Pawelec, et al., 2009). Studies done in the United States population revealed a seroprevalence of CMV in those aged 80 years or older of more than 90%, the mean age of infection being 28.6 years. On average, one infected person transmitted the virus to nearly two susceptible people (Staras, et al., 2006; Colugnati, et al., 2007).

1.5.3 Pathology

Immunocompetent patients usually have no symptoms during primary infection and reactivation. Mainly diagnosed are mononucleosis with fever, myalgia, adenopathy, splenomegaly (Colugnati, et al., 2007; Crough & Khanna, 2009). 10% of congenitally infected children are symptomatic at birth and of those who are asymptotic, 10-15% will develop symptoms over month or years. Among this group, CMV is a leading cause of hearing loss, vision loss, and mental retardation (Colugnati, et al., 2007). Aside of that, the main problems CMV is causing is as a serious opportunistic infection of immunocomprimised patients. Although the introduction of highly active antiretroviral therapy (HAART) for HIV treatment, resulting in decreased immune suppression, seriously reduced HCMV-associated diseases, it continues to be problematic and was suggested to accelerate the progress of AIDS and death. In solid organ transplantation (SOT) recipients, HCMV is the major infectious pathogen, effecting both graft survival and causing morbidity and mortality of the patient (Crough & Khanna, 2009). Similarly, in patients obtaining hematopoietic stem cell transplantation, HCMV is a major source of complications, associated with chronic graft-versus-host disease and increased risk of secondary bacterial and fungal infections (Boeckh & Ljungman, 2009). Depending on the conditions of CMV activation, clinical symptoms of HCMV disease vary, with febrile illness with leukopenia and malaise, multi-organ disease including gastroenteritis, pneumonitis, retinitis, and hepatitis being just a few examples (Crough & Khanna, 2009).

1.5.4 HCMV life cycle

After a primary infection, CMV is able to establish a latent, life-long infection with spontaneous, periodic reactivation (Colugnati, et al., 2007; Crough & Khanna, 2009). In vivo, CMV-derived DNA was found in monocytes, macrophages, neutrophils, lymphocytes and endothelial cells. (Sia & Patel, 2000) During HCMV outbreak the cellular tropism is wide including endothelial cells, various leukocyte populations, epithelial cells, hepatocytes, smooth muscle

cells, and fibroblasts (Sinzger, et al., 1995; Razonable, 2008) whereupon the organ distribution is stunning: For example, all tested organs (adrenal gland, bone marrow, diencephalon, heart, kidney, liver, lung, pancreas, placenta, small bowel and spleen) were found to carry HCMV in case of a congenitally infected neonate (Bissinger, et al., 2002). The mechanism where and how latency is established remains to be elucidated. The discovery that hematopoietic precursors ($CD34^+$ cells) isolated from bone-marrow carry CMV and pass it over to monocytes (but not B- and T- cells) during differentiation, maybe as episomal molecules, hints at a possible mechanism (Sinclair, 2008).

Following CMV infection, the viral genome is expressed sequentially. During the first two hours, the immediate early (IE) genes are expressed followed by the early (E) (< 24 hour) and late (L) (> 24 hour) genes (Stinski, 1978). While CMV is known as a slowly replicating virus, *in vivo* analysis revealed that HCMV replication is a highly dynamic process and under permissive conditions, the doubling time in blood likely takes about one day (Emery, et al., 1999). After interaction of viral glycoproteins with cell surface receptors leading to attachment of the virus, penetration into a target cell occurs. The nucleocapsid is released into the cytoplasm and subsequently translocated to the nucleus where the viral DNA is released. Expression of IE genes starts inducing viral DNA transcription and replication. Viral proteins are imported from the cytoplasm to the nucleus and the capsid reassembles, encapsulating the CMV genome. After nuclear egress, assembly with the envelope in the cytoplasm and virion release by exocytosis completes the CMV life-cycle (Crough & Khanna, 2009).

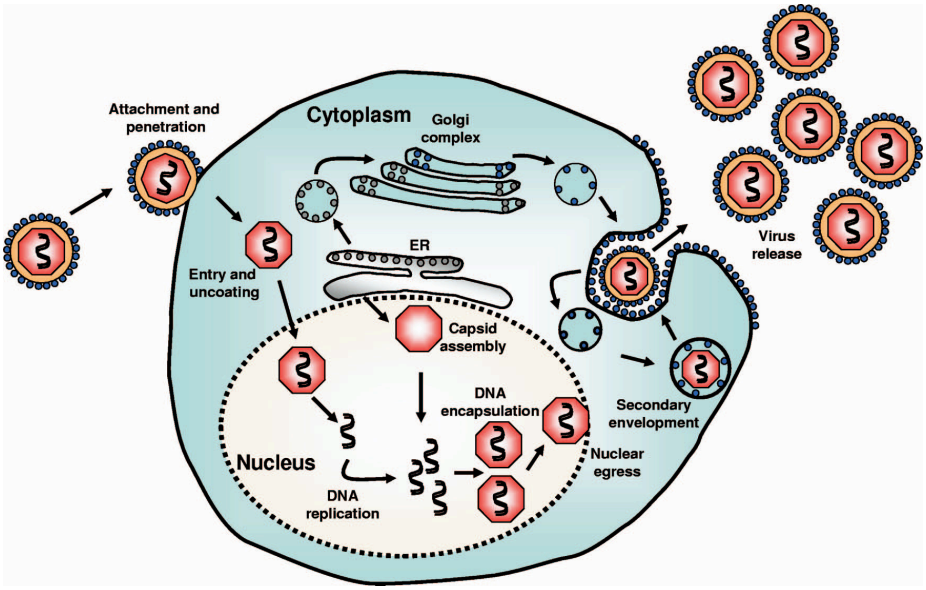

Figure 1.10 HCMV life cycle
Upon HCMV infection, the capsid is released to the cytoplasm and subsequently translocated to the nucleus where the viral DNA is released, inducing viral gene expression. Viral proteins, previously translated and imported to the nucleus, encapsulate the replicated viral DNA. After nuclear egress, the capsid is enveloped and the fully assembled virus gets exocytosed, resulting in free HCMV (Crough & Khanna, 2009).

1.5.5 HCMV immune modulation of cytotoxic lymphocyte-mediated responses

Among the herpesviruses, CMV encodes the greatest number of genes committed to altering magnitude and quality of both innate and adaptive immune responses. The mechanisms are diverse including (but not restricted to) molecular mimicry of cell surface receptors, cytokine homologues and inhibition of apoptosis (Miller-Kittrell & Sparer, 2009).

1.5.5.1 Modulation of HLA class I expression

The ability of the immune system to monitor "self" and recognize pathogens with the same molecular system makes it a tightrope walk for CMV to reduce MHC class I expression to escape from cytotoxic T cell responses but then keeping sufficient MHC class I surface expression to prevent "missing self" responses by NK cells.
A multitude of viral unique short (US) proteins (US2, US3, US6, US10, US11) interfere by concerted action with MHC class I formation, transportation and expression, using mechanisms like retention of fully assembled proteins in the ER, redirecting nascent MHC class I heavy chains to the cytosol for degradation and inhibiting TAP-dependent protein translocation to the ER. (Lin, et al., 2007) At the same time, the non-classical HLA-E molecule is retained on the surface: The leader sequence of HCMV glycoprotein UL40 contains exactly the same peptide as is derived from HLA-C and assembled with HLA-E. Loading of the virus-derived peptide by a TAP independent mechanism enables CMV to not only stabilize but upregulate surface expression of HLA-E which was found to protect against cytolysis by $NKG2A^+$ NK cells (Tomasec, et al., 2000). To further compensate the MHC class I downregulation, HCMV encodes the MHC class I homologues gpUL18 and gpUL142. The better characterized UL18 was shown to associate with β_2-microglobulin, bind a mixture of endogenous proteins with similar characteristics to those that bind HLA class I and it is a ligand for the inhibitory ILT2 (CD85j) bound with >1000-fold higher affinity than host MHC-class I (Chapman, et al., 1999; Wills, et al., 2005).

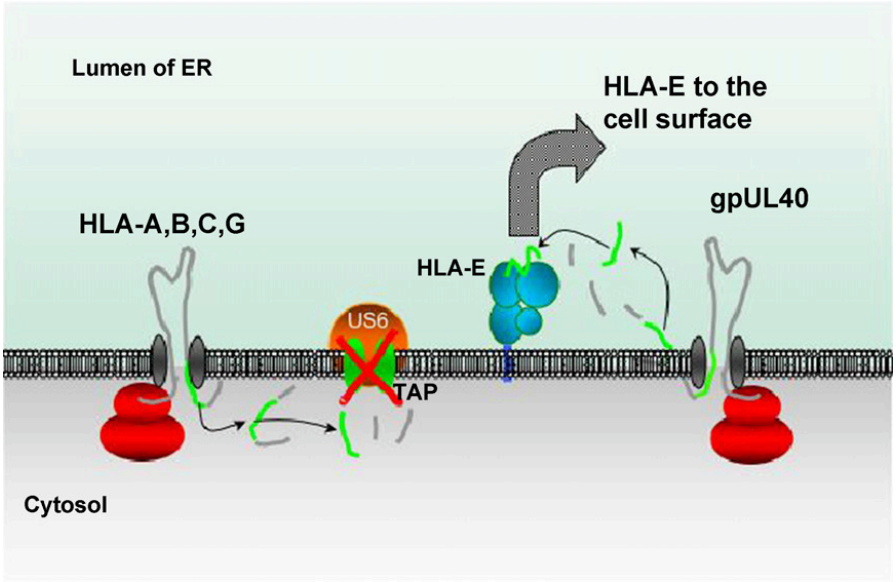

Figure 1.11 Modulation of HLA-E surface expression by HCMV
The HCMV encoded US6 protein prevents TAP-dependent protein translocation to the ER, which reduces MHC class I surface expression due to the lack of MHC-binding peptides. To counteract a "missing self" response, gpUL40 derived peptides, which reach the ER in a TAP-independent manner, bind to HLA-E, driving its surface expression (Wilkinson, et al., 2008).

1.5.5.2 Modulation of NKG2D ligand (NKG2DL) surface expression

Immunosurveillance of HCMV includes upregulation of ligands for the activating NK cell and co-activating T cell receptor NKG2D: MICA, MICB, UL16-binding proteins (ULBP1-4), RAET1G and RAET1E (Eagle & Trowsdale, 2007; Wilkinson, et al., 2008; Jonjic´, et al., 2008). It is therefore no surprise that HCMV has several immunoevasion strategies aimed at NKG2D to counteract the potent ligand-upregulating action of particularly its immediate early (IE) proteins (Wilkinson, et al., 2008). So far, two proteins and a micro-RNA were identified to act on the prevention of cell-surface expression of NKG2DLs: UL16 retains MICB, UL16BP1 and UL16BP2 in the endoplasmatic reticulum (ER) (Welte, et al., 2003; Wu, et al., 2003; Wilkinson, et al., 2008) whereas MICA expression is impeded by UL142 (Chalupny, et al., 2006). The HCMV encoded micro-RNA miR-UL112 selectively downregulates MICB expression (Stern-Ginossar & Elefant, 2007). Cooperative targeting of NKG2DL surface expression through protein- and miRNA-mediated mechanisms may thwart NKG2D mediated "stress-signaling" during viral infection.

1.5.5.3 Modulation of the cytokine and chemokine system

The homologue for the immunosuppressive cytokine IL-10 (cmvIL-10) binds the cellular IL-10 receptor with the capacity to induce an anti-inflammatory response. Cmv-IL-10 was shown to have a similar antiproliferative capacity as cellular IL-10 (Spencer, et la., 2002). UL147 mimics

host IL-8 and binds with high affinity to the cognate receptor CXCR2. The beneficial effect of CXCR2 signaling could either be disruption of normal cell migration patterns or attraction of lymphocyte subsets not capable to efficiently clear virus (Penfold, et al., 1999). From the four HCMV chemokine receptor homologues, US28 is the best characterized. It is most similar to CCR1 and binds the CC chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CCL7 (MCP-3) but also CX_3CL1 (fractalkine). Constitutive internalization of US28 with bound chemokines could lead to immune modulation by the removal of the chemokines from the cellular environment. US28 is also a functional receptor mediating downstream signaling via G-proteins, eliciting Ca^{2+} mobilization and MAP kinase (ERK2) activation. The utilization of cellular G proteins could manipulate cellular cytokine-mediated responses to the benefit of CMV (Billstrom, et al., 1998; van Cleef, et al., 2006). Another mechanism to block chemokine signaling is to bind and occupy them in solution as found for the secreted RANTES decoy receptor US21.5 (Wang, et al., 2004).

1.5.5.4 Other important mechanisms supporting HCMV immunoevasion

Antibody-mediated immunity is inhibited by the expression of two FcR binding proteins, which may protect from ADCC and complement activation. The incorporation of the cellular proteins CD55 and CD59 in the virion and upregulation of these proteins on the host cell surface protects virion and cell from complement-mediated lysis (Miller-Kittrell & Sparer, 2009). To delay the death of HCMV infected cells, at least two anti-apoptotic proteins directly interfering with the apoptotic signaling pathways were found to date (Goldmacher, et al., 2005).

Collectively, all these manipulative strategies of CMV exceed the capabilities of the human host to prevent or clear an infection, but as long as the immune system is not debilitated, coevolution has led to mechanisms which, in the majority of cases, result in harmless coexistence enabled through “arranged” immunoevasion and immunosurveillance. Even so, HCMV is a serious pathogen that puts a burden on the immune system and despite intense research, we still miss a treatment that can shift the balance towards the benefit of the host, enough to clear HCMV.

1.6 Aims

Surface protein expression is a major aspect influencing the property of cells and has been successfully used to identify functionally distinct subsets. There is increasing evidence that CD94/NKG2 receptors, expressed on NK and T cells, are important during various immune reactions but to date, it is not clear to whcih extent these receptors cotribute to cellular functions and if their expression defines heterogeneous lymphocyte populations. The amis included:

1. To define subpopulations characterized by the expression pattern of CD94/NKG2 receptor isoforms and to assess the frequency and distribution of CD94/NKG2 receptors within isolated PBMC of different individuals.

2. To correlate CD94/NKG2 receptor expression with known NK and T cell subpopulations.

3. To analyze the mitotical and functional activity of CD94/NKG2 receptor-expressing cells upon encounter of target cells expressing the cognate ligand HLA-E and in the context of CMV infection.

2. Materials & Methods

2.1 Cell culture techniques

2.1.1 Cell lines and primary cells

LCL 721.221. (MHC class 1a deficient EBV-transformed B lymphoblastoid cell line kindly provided by Prof. Miguel Lopez-Botet, Universitat Pompeu Fabra, Barcelona), K562 (human myelogenous leukemia cell line), MRC-5 (Human embryonic lung fibroblasts obtained from American Type Culture Collection (ATCC) or kindly provided by Prof. Hartmut Hengel, Heinrich Heine Universität, Düsseldorf) and isolated PBMC were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat inactivated fetal calf serum (FCS, Sigma-Aldrich), 2 mM L-glutamine (200 mM solution, Sigma-Aldrich), 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (Pen/Strep Fungizone Mix, Bio Whittaker) and 1 mM sodium pyruvate (Sigma-Aldrich) (referred to as complete medium). Medium was sterile-filtered (0.22 µm pore size Stericup® Filter Units, Milipore) after addition of supplements. For TB40 stock preparation (2.1.5), MRC-5 cells were cultured in DMEM / High Glucose medium (Fisher Scientific) supplemented in an analogous manner.
LCL 721.221.AEH cells (HLA-E*0101 transfected, kindly provided by Prof. Miguel Lopez-Botet, Universitat Pompeu Fabra, Barcelona) were cultured in complete RPMI 1640 supplemented with 200 µg/ml hygromycin B (Invitrogen).
Cell lines were screened and shown to be negative for Mycoplasma contamination by polymerase chain reaction (mycoplasma detection kit, Roche)
All cells were maintained in a humidified cell-culture incubator at 37°C in 5% CO_2 (referred to as standard conditions)
HUVEC (human umbilical vein endothelial cells, isolated in our laboratory) were seeded onto gelatine-coated tissue culture flasks or plates in M199 medium (Lonza) supplemented with 20% heat inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (Pen/Strep Fungizone Mix, Bio Whittaker), 12 μg/ml Endothelial Cell Growth Supplement (PromoCell), 2 mM L-glutamine (Sigma) and 5 U/ml heparin (Fisher Scientific) (referred to as complete medium). Medium was sterile-filtered (0.22 µm pore size Stericup® Filter Units, Milipore) after addition of supplements.

2.1.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were obtained from fresh heparinized whole peripheral blood of healthy donors by centrifugation on Ficoll-Paque™ PLUS (Amersham) using standard procedures. All steps are done on ice or at 4°C using pre-cooled solutions. In brief, peripheral blood is diluted 1:1 with Calcium- and Magnesium-free Phosphate Buffered Saline (PBS, Bio Whittacker) and 30 ml

cell suspension is carefully overlaid on 15 ml Ficoll-Paque™ in a 50 ml Falcon™ tube (BD). After 30 min centrifugation at 700 x g without brake, the buffy coat is transferred to a fresh tube and diluted 1:2 with PBS. The cell suspension is centrifuged at 250 x g for 10 min, the pellet is resuspended in 50 ml PBS and centrifuged again 10 min at 100 x g (platelets stay in solution). According to the manufacturer, this procedure leads to isolation of lymphocytes and monocytes whereas other components (including granulocytes, erythrocytes & platelets) are largely removed. PBMC are resuspended in complete RPMI 1640 medium or FACS buffer (see below) for in vitro culture or analysis, respectively.

2.1.3 Magnetic activated cell sorting (MACS)

NK cells were negatively selected from PBMC by magnetic activated cell sorting (MACS) using an NK Isolation Kit (Miltenyi Biotec, cat. 130–092–657) following the manufacturer's instructions. In brief, PBMC are incubated with a mix of biotin-labeled antibodies recognizing proteins expressed on various immune cells but not on NK cells. Incubation with biotin-binding magnetic beads allows to remove antibody-bound cells by loading onto a column placed in a magnetic field. Magnetically labeled cells are retained and NK cells remain in the flow-through.

2.1.4 Co-culture of PBMC with LCL 721.221. cells

LCL 721.221. or 721.221.AEH cells were irradiated at 30 gray at a cell density ≤ 10^7 per ml. 721.221.AEH were transferred to medium without hygromycin B at least 24 hours before irradiation. 3 x 10^6 freshly isolated PBMC were incubated alone or with 1 x 10^6 irradiated cells per well of a 24 well plate in complete RPMI 1640. The culture medium was partially exchanged every 2-3 days by carefully aspirating 50%, replacing it with fresh complete RPMI 1640 and resuspending the cells. Alternatively, cells were resuspended, split 1:1 and supplemented with fresh medium commensurate to the transferred suspension volume (therefore equalling 50% medium exchange).

2.1.5 Co-culture of PBMC with HCMV infected fibroblasts

HCMV infected and mock treated MRC-5 cells were co-cultured in 24-well plates using complete RPMI 1640 medium. 24 hours post infection, 2 x 10^6 freshly isolated PBMC were added per well to the culture supernatant. The culture medium was partially exchanged every 1-2 days by carefully aspirating an equal portion of each well, replacing it with fresh complete RPMI 1640 and resuspending the cells. Starting three days after the co-incubation, all cultures were supplemented with 10 international units (IU) recombinant human IL-2 (R & D Systems) per ml.

2.2 Working with HCMV

2.2.1 Infection of MRC-5 cells and HUVEC with HCMV

MRC-5 cells (passage 8-17) or HUVEC (passage 3-5) were seeded in tissue-culture plates in the respective complete medium (see 2.1.1) until 70 % confluence and infected with AD169 (MRC-5) or TB40 (MRC-5, HUVEC) (both lab strains kindly provided by Prof. Hartmut Hengel, Heinrich Heine Universität, Düsseldorf) by pipetting the virus-containing suspension in the cell culture supernatant using a multiplicity of infection (MOI) of 1, 2.5 or 5. Plates were centrifuged 2 x at 700 x g for 15 minutes at 37°C, turning the plates 180° in between the runs. Cells were incubated under standard conditions and after a total of 2 hours virus exposure, the culture supernatant got aspirated and replaced with fresh complete medium. Mock-treated cells were proceeded in parallel and received PBS instead of the virus-containing suspension.

2.2.2 Preparation of a HCMV (TB40) stock

A confluent 6-well plate MRC-5 cells (passage 17) was infected with TB40 as described in 2.1.4 with the exception that the virus-containing medium was not exchanged after 2 hours. Four days post infection, supernatant and cells (using a cell-scraper) were collected and transferred to two confluent T175 flasks MRC-5 cells (passage 17). After one week incubation, cells and supernatant were collected again and distributed equally to 21 confluent T175 flasks MRC-5 cells (passage 11). Fresh medium (complete DMEM) was added if the medium-color turned towards orange. Nine days later, cells and supernatant were collected and centrifuged at 3500 x g for 20 min at 15°C to pelletize cells and debris. The supernatant was transferred to fresh tubes, centrifuged at 25 000 x g for 3 hours at 15°C (to pelletize the virus) and resuspended in 10 ml ice-cold PBS. A pre-cooled (-20°C) douncer was used to homogenize the suspension by carefully inserting and pulling out the pestle 20 times. The virus suspension was carefully overlaid on a 20 ml ice-cold sorbitol cushion (20% sorbitol in dH_2O, 50 mM Tris ph 7.4, 1 mM $MgCl_2$, 100 μg/ml bacitracin (Sigma-Aldrich); sterile filtered) and centrifuged 1 hour at 60 000 x g at 10°C. The pellet was resuspended in 3 ml PBS and homogenized with a douncer as described above. 20 or 30 μl aliquots were prepared and stored at -80°C.

2.2.3 HCMV (TB40) titration

MRC-5 cells were cultured in a 96 well flat-bottom plate until 90% confluence. The virus stock was pre-diluted 1:10 with medium and added to the wells in 10-fold serial dilution steps on a 200 μl scale followed by 2 x 15 minutes centrifugation at 700 x g at 37°C. 72 hour post infection, the medium was removed and 100 μl methanol (-20°C) added per well to fix the cells. After 10 min incubation on ice, methanol was aspirated, the plate was air-dried and washed

once with PBS. For CMV detection, cells were incubated with a monoclonal mouse anti-CMV antibody-mix comprised of clones CCH2 (recognizing an early protein) and DDG9 (recognizing an immediate early protein) (Dako, kindly provided by Prof. Hartmut Hengel). The primary antibody-mix was diluted in PBS with 1% skim milk, added to the fixed cells and incubated for one hour at room temperature. After washing twice with PBS, cells were incubated with horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG antibody (GE Healthcare, cat. NA931V). Antibody-bound cells were detected using 3-amino-9-ethyl-carbazol (AEC, kindly provided by Prof. Hartmut Hengel) which reacts with HRP, producing a red-brown precipitate. The reaction was stopped by removal of the substrate solution and washing once with PBS as soon as stained cells were observed. CMV infected cells were counted and the number of infectious units (PFU, plaque forming units) per ml virus stock was calculated. Titration and detection was performed in triplicates.

2.2.4 CMV infection status of PBMC donors

Blood samples were taken into non-heparinized, citrate-containing tubes to prevent coagulation and to stabilize serum antibodies. Test of CMV infection state of each donor was performed by the "Clinical Institute for Virology" of the "Medical University of Vienna" via PCR and CMV-IgG and -IgM ELISA.

2.3. Flow cytometry

2.3.1 Preparation of cells

Adherent cells (HUVEC, MRC-5) were detached by washing once with PBS and incubation with trypsin-EDTA (Sigma-Aldrich). The action of trypsin was stopped by the addition of trypsin-inhibitor (Sigma-Aldrich) or FCS followed by resuspension of the cells with culture medium.

Cells growing in suspension (721.221, 721.221.AEH, K562, PBMC) as well as suspended adherent cells were transferred to 50 ml Falcon™ tubes and centrifuged 10 min 200 x g at 4 °C, washed once with ice-cold PBS and resuspended in ice-cold FACS buffer (PBS + 2% FCS + 0.1% NaN_3).

To inactivate CMV, virus exposed (and mock-treated) cells were washed with PBS, incubated for 10 min on ice with CellFix (BD Biosciences), washed again and resuspended in FACS buffer.

Cells were counted in a haemocytometer and adjusted to 1-5 x 10^5 per 50 μl with FACS buffer.

2.3.2 Monoclonal antibodies

Specificity (all human)	Clone	Conjugate	Source
CD3	UCHT1	APC	AbD Serotec
CD3	UCHT1	FITC	Abcam
CD16	3G8	PE	BD Biosciences
CD56 (NCAM)	NCAM16.2	FITC	BD Biosciences
CD62L (L-selectin)	DREG-56	PE	BD Biosciences
CD94	DX22	FITC	AbD Serotec
CD107a (LAMP-1)	H4A3	FITC	BD Biosciences
CD158 (KIR)	180704	Fluorescein	R & D Systems
CMV encoded proteins	CCH2 and DDG9		Dako *
HLA-DR	L243	PE	BD Biosciences
HLA-E	MEM-E/08		Abcam
MHC class I	W6/32		*
NKG2A (CD159a)	Z199	PE	Beckman Coulter
NKG2C	134591	APC	R & D Systems
NKG2C	134591	PE	R & D Systems
NKp46	9E2/NKp46	APC	BD Biosciences

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin
* kindly provided by Prof. Hartmut Hengel, Heinrich Heine Universität, Düsseldorf.

2.3.3 Antibody staining and analysis by flow cytometry

Cells and solutions are kept on ice and in dark throughout the entire procedure. 50 μl aliquots of suspended cells were transferred to 96-well V-bottom plates (Greiner) and incubated 10 min with 2 μl Fc receptor blocking reagent (Miltenyi) followed by the addition of saturating concentrations of primary monoclonal antibodies (sell 2.2.2). For multicolor staining, differentially fluorochrome-conjugated (APC, FITC, Fluorescein, PE) antibodies were simultaneously added to the samples. After 30 min incubation, 100 μl FACS buffer was added, cells were centrifuged 5 min at 600 x g and the supernatant was discarded. The pellet was resuspended in 150 μl FACS buffer, centrifuged again and finally resuspended in 25 to 100 μl FACS buffer (depending on the total cell number per sample) for analysis by flow cytometry. For indirect immunofluorescent staining with unlabeled primary antibodies, cells were subsequently incubated with a polyclonal, FITC labeled anti-mouse Ig antibody (Dako) using the same staining-procedure. A FACS Calibur machine (Becton Dickinson) was used for measurement and Cell Quest Pro software (Becton Dickinson) for analysis. Cells were incubated for 5 min on ice with 1 μl 7-amino-actinomycin D (7AAD, eBioscience) before measurement to examine the viability.

2.3.4 Recombinant human Fc-FITC staining

CMV infected and mock treated HUVEC and MRC-5 cells were stained with recombinant human Fc-FITC (Rockland, kindly provided by Prof. Hartmut Hengel, Heinrich Heine Universität, Düsseldorf) the same way as described for primary labeled antibodies with the exception that cells were not incubated with Fc receptor blocking reagent.

2.4 CD107a surface mobilization assay

CD107a (LAMP-1) is found on the cell surface of NK and T cells upon degranulation and its surface expression correlates with the cytolytic and/or cytokine producing activity (Betts, et al., 2003; Alter, et al., 2004).
Freshly isolated or co-cultured total PBMC or MACS sorted NK cells were used as effector cells and 721.221., 721.221.AEH and K562 as targets cells ("no target" control cells received medium). Effector (E) and target (T) cells were incubated in complete RPMI 1640 growth medium at a ratio of E:T = 10:1 using ≥ 1 x 10^6 effectors per ml in 24 or 96 well plates (Greiner). Immediately after the start of the co-incubation, 20 μl FITC labeled anti-CD107a antibody (BD Biosciences) was added per ml culture medium. After 1 hour incubation in a cell culture incubator at standard conditions (37°C, 5% CO_2), 6 μg/ml monensin (Sigma-Aldrich) was added to prevent the degradation of reinternalized CD107a proteins in endocytic vesicles (Alter, et al., 2004) and cells were incubated for an additional 5 hours. Finally, the cells were stained with APC or PE labeled monoclonal antibodies and analyzed by flow cytometry as described in chapter 2.3.

3. Results

Nk cells are constituted by complex mixtures of cells expressing varying combination of NK receptors. Furthermore, NK receptors can be expressed on a fraction of T cells. In the first part of this work it was attempted to define subpopulations of NK and T cells distinguished by their expression of CD56 and the CD94/NKG2 heterodimeric receptors. The second part was aimed to functionally characterize these cell-subpopulations.

3.1 Analysis of NKG2A and NKG2C expression within PBL subpopulations

3.1.1 Lymphocyte gating and identification of PBL subpopulations by surface protein expression

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized whole blood by density gradient centrifugation after overlay on Ficoll-Paque. PBMC stained with fluorescent labeled antibodies specific for various surface proteins were analyzed using flow cytometry. Cells were plotted in a forward (FSC) versus side (SSC) scatter dot-blot, which allows to electronically gate on the lymphocytic population because of their characteristic light scattering properties as described by Loken et al. (1990). FSC corresponds to cell size and SSC corresponds to granularity. Cytometer settings, suitable to distinguish lymphocytes from monocytes do not allow to simultaneously display neutrophils, basophils and eosinophils because they are bigger and much more granular. The viability of isolated PBMC was confirmed by incubation with 7-amino actinomycin D (7aad) which stains dead and apoptotic cells (Philpott, et al., 1996) (Fig. 3.1).

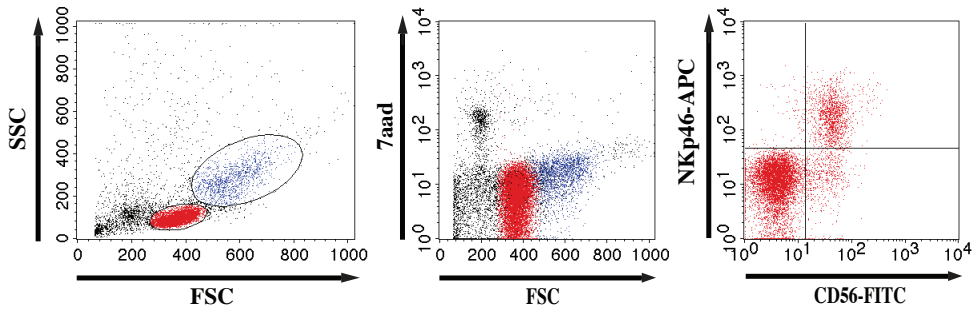

Figure 3.1 Lymphocyte gating in flow cytometry
PBMC are gated on the lymphocytic fraction (indicated in red) using a light scattering gate to distinguish them from monocytes (indicated in blue). Incubation with 7aad, which stains dead and apoptotic cells, is used to confirm cell viability. Fluorescent labeled antibodies enable to identify lymphocyte populations defined by surface protein expression as indicated in the right dot blot using APC labeled anti-NKp46 and a FITC labeled anti-CD56 antibodies.

To distinguish PBL subpopulations, specific antibodies against CD3, CD56 and NKp46 were used. Natural killer cells (NK cells) are usually identified as being either $CD3^-$ and $CD56^+$ or $NKp46^+$ and $CD56^+$. NKp46 alone is considered to be a specific marker for NK cells but functional subsets can be defined by the pattern of CD56 expression. Because of that, the small population of $NKp46^+$ $CD56^-$ NK-cells is not considered in the current studies unless otherwise noted (Fig. 3.2 A). Two major NK cell subpopulations defined by the strength of CD56 expression are referred to as $CD56^{bright}$ or $CD56^{dim}$ cells according to their strong or weak staining for CD56, respectively. The CD16 and CD62L expression patterns indicate a functional difference between these populations (see chapter 1.2.4) and confirm the appropriate use of gates to determine the frequency of subpopulations and to analyze expression of other surface proteins within the subpopulations (Fig. 3.2 B). A small fraction of $CD56^+$ cells co-expresses CD3, indicating that these cells belong to the T cells compartment and thus dividing $CD56^+$ cells in an NK and T cell subset. The distribution of NK and T cells within PBL as well as the subsets defined by CD56 expression is shown in Table 3.1. The different cellular components of PBL varied between different individuals but the most prominent cells were T cells, regularly giving values of about 70% of PBL. On average, only 9% of T cells express CD56, making NK cells to the major CD56 expressing subset in most individuals, with the exception of donor 2. NK cells typically comprise on average 16.5% of PBL with an high variation between individuals ranging from 4% to 24.5%.

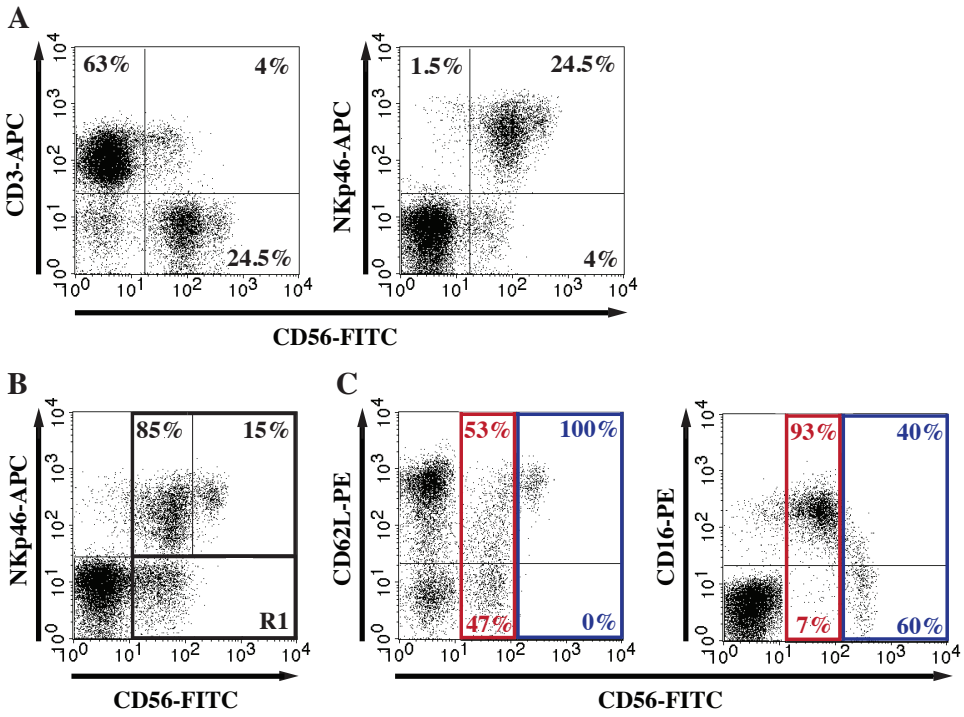

Figure 3.2 Distribution of NK and T cell markers on PBL
A, To identify T cells and NK cells within lymphocytes, cells are stained with anti-CD3-APC and anti-CD56-FITC or anti-NKp46-APC and anti-CD56. NK cells are identified as being either $CD3^-$ $CD56^+$ or $NKp46^+$ $CD56^+$. T cells are $CD3^+$ and some also $CD56^+$. *B*, *C*, $CD56^{bright}$ (15%, blue) and $CD56^{dim}$ (85%, red) NK cell subpopulations differentially express CD62L and CD16. $CD56^+$ non-NK cells are removed by electronic gating using R1 indicated in (B).

	T cells			NK cells		
Donor	% of PBL	% CD56-	% CD56+	% of PBL	% CD56 dim	% CD56 bright
1	76.5	91.0	9.0	11	91.0	9.0
2	74	88.0	12.0	4	87.5	12.5
3	67	97.0	3.0	21	95.0	5.0
4	71	93.0	7.0	14	93.0	7.0
5	65	83.0	17.0	23	85.0	15.0
6	68.5	93.5	6.5	24.5	87.5	12.5
7	68	91.0	9.0	18	94.5	5.5
Mean	70 +/- 4	91 +/- 4.5	9 +/- 4.5	16.5 +/- 7	90.5 +/- 4	9.5 +/- 4

Table 3.1 Distribution of NK and T cell subpopulations in PBL
PBMC of 7 healthy donors are stained with specific antibodies against CD3 and CD56 to identify PBL subpopulations in flow cytometry. NK cells are defined by NKp46 and CD56 staining, T cells by CD3.

3.1.2 Co-expression of CD94 with NKG2 receptor isoforms

NKG2A and NKG2C dimerize with the invariant CD94 chain in order to get expressed on the cell surface. The unusual appearance of the $CD94^+$ $NKG2^+$ cell population on the diagonal indicates that the amount of NKG2A or NKG2C on the cell surface correlates to a large extent with the amount of CD94, reflecting the cell surface co-expression as heterodimers (Fig. 1.2 A). It is not clear weather the 0.5% of cells found to express NKG2 isoforms without CD94 really exist or if they are artifacts due to an unspecific staining of the antibodies. The expression of NKG2A and NKG2C is not mutually exclusive as a small population expresses both receptors. Thus, by this analysis Nk cells can be subdivided in 4 subpopulations, a $NKG2A^+$, a $NKG2C^+$, a $NKG2A^+/NKG2C^+$ double positive and a double negative population. Other NKG2 receptor isoforms, such as NKG2E and NKG2H, which are thought to have to dimerize with CD94 in order to reach the cell surface, could only be form small separate subpopulations since usually the number of CD94 positive cells is close to the sum of NKG2A and NKG2C positive cells, as shown for a representative PBL donor in figure 3.3. However, as previously suggested (Brostjan, et al., 2002), it is possible that NKG2E and possibly also NKG2H might be co-expressed to a lower extent with the NKG2C subpopulation. Since specific antibody reagents for these NKG2 isoforms are still missing, this possibility can not be evaluated yet.

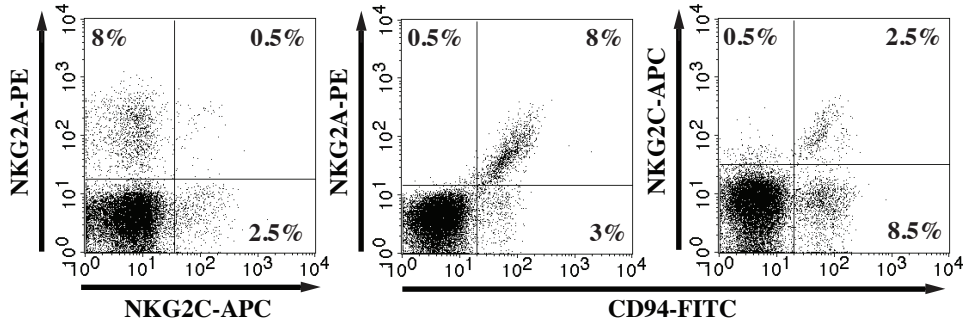

Figure 3.3 Co-expression of CD94/NKG2A and CD94/NKG2C
PBMC were triple-stained with CD94, NKG2A and NKG2C specific antibodies. Analysis of PBL in flow cytometry shows co-expression of NKG2-A and -C with the invariant CD94 chain.

3.1.3 NKG2A and NKG2C expression in PBL subpopulations

The expression of CD94/NKG2-A or -C by PBL is restricted to T and NK cells (Fig. 3.4) with NKG2A being the predominant receptor isoform in both fractions. The major cell type expressing NKG2 receptors are NK cells. This is true when considering the total cell number of CD94/NKG2 expressing cells as well as the percentage of CD94/NKG2 expressing cells within the NK as compared to the T cell population. This seems to hold true as long as the PBL donor has usual NK cell numbers. Donor number 2, which had only 4% NK cells, was the only observed case where the majority of CD94/NKG2 expressing cells were T cells.

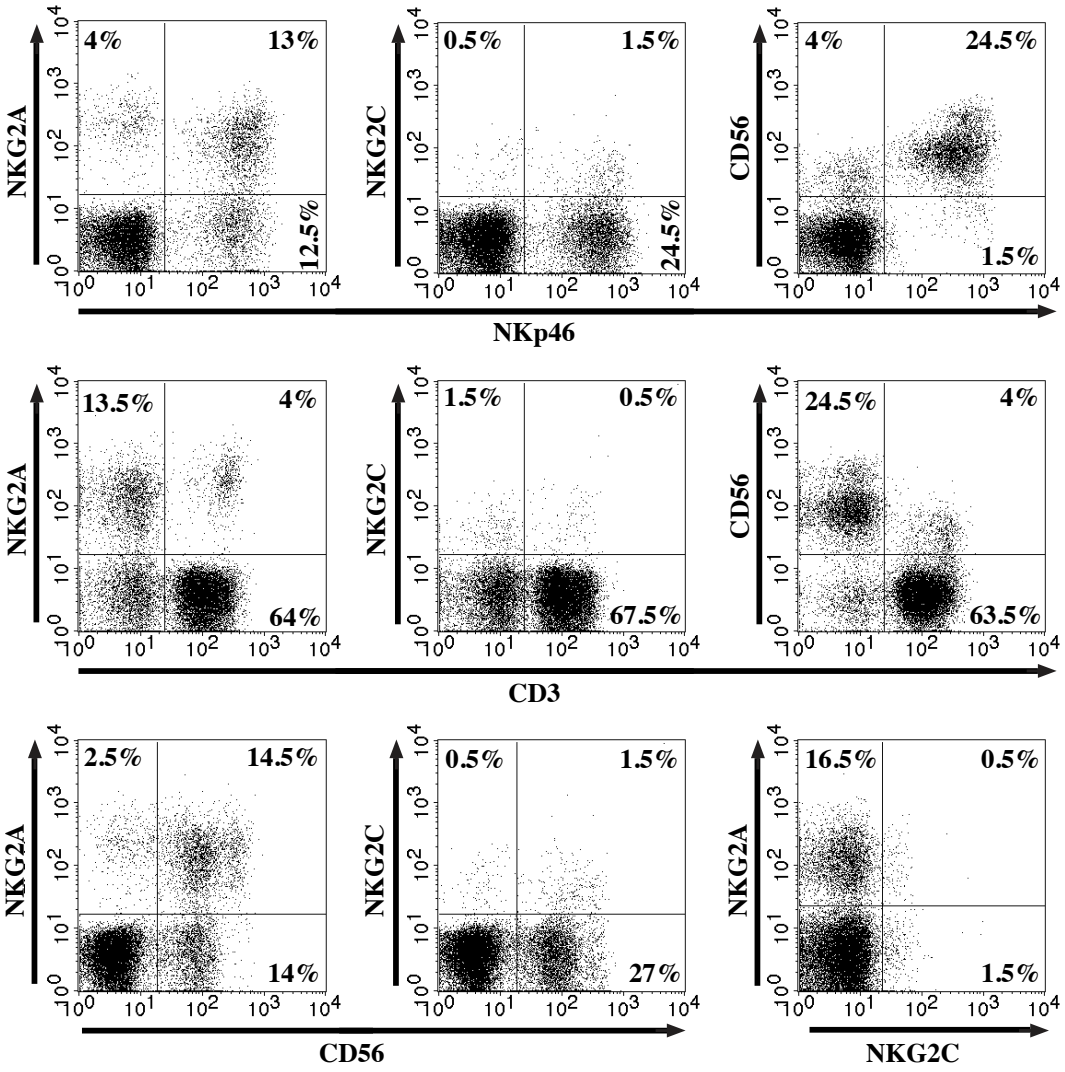

Figure 3.4 NKG2A and NKG2C expression in PBL subpopulations
Exemplary PBL analysis of NKG2A and NKG2C expression on NKp46, CD3 and CD56 expressing cells.

Simultaneous staining of PBL with specific antibodies conjugated to different fluorochromes (APC, FITC, PE) allows to correlate the expression of proteins to each other. NK and T cell sub-populations, defined by CD56 expression were analyzed for the distribution of CD94/NKG2-A and -C. Both isoforms show a preferential expression on $CD56^{bright}$ as compared to $CD56^{dim}$ NK cells and on $CD56^{+}$ as compared to $CD56^{-}$ T cells (**figure 3.5**). This pattern was true for every donor investigated (n = 7), although the extent varied:

% CD56 bright $NKG2A^{+}$ NK-cells : % CD56 dim $NKG2A^{+}$ NK-cells = 1.8 +/- 0.3 (range: 1.5 - 2.3)
% CD56 bright $NKG2C^{+}$ NK-cells : % CD56 dim $NKG2C^{+}$ NK-cells = 4.9 +/- 3.6 (range: 3.0 - 12.5)

% CD56+ $NKG2A^{+}$ T cells : % $CD56^{-}$ $NKG2A^{+}$ T cells = 8.9 +/- 4.4 (range: 4.6 - 17.0)
% CD56+ $NKG2C^{+}$ T cells : % $CD56^{-}$ $NKG2C^{-}$ T cells = 9.0 +/- 7.6 (range: 2 - 24)

The expression of NKG2A within the NK cells population was found similar for each donor whereas NKG2C shows much higher alterations with regard to the percentage of expressing cells. T cells expressing NKG2 isoforms were generally found to represent only a small sub-population but the correlation with CD56 expression was strong.

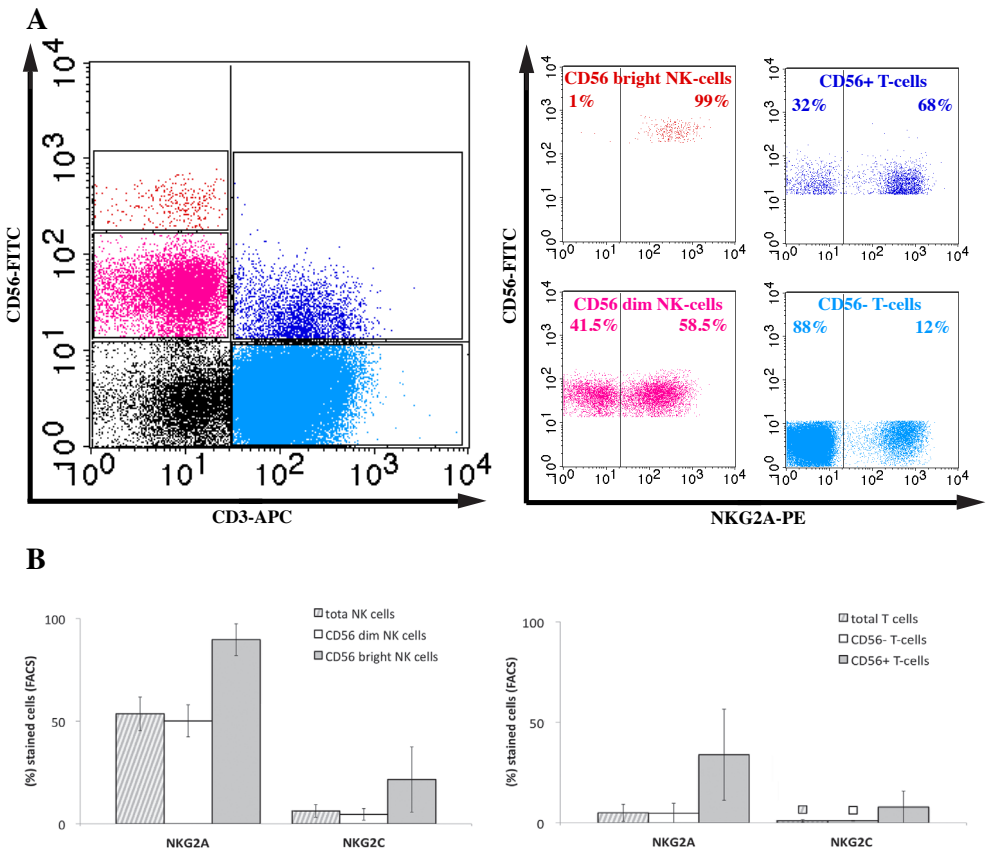

Figure 3.5 CD56 expression correlates with NKG2-A and -C expression on NK and T cells
A, Exemplary PBL staining, indicating the gates used for the analysis of NKG2A and NKG2C expression within PBL subpopulations; $CD56^{bright}$ NK (dark red), $CD56^{dim}$ NK (pink), $CD56^{+}$ (dark blue) and CD56- (light blue) T cells. Cells were triple-stained with CD3-APC, CD56-FITC and NKG2A-PE or NKG2C-PE. *B*, Mean values and standard deviation of 7 donors analyzed for NKG2-A and -C expression.

Similar to the lectin-like NKG2 receptors, killer immunoglobin-like receptors are exclusively expressed on T and NK cells and comprise inhibitory and activatory members. Because of these similar properties, it was of interest to test, if NKG2 and KIR expression is linked. Figure 3.6 shows one out of three PBMC donors analyzed. If no linkage between NKG2A and/or NKG2C and KIR expression takes place, one would expect that expression of NKG2-A and -C among KIR^+ cells is as frequent as expression of NKG2A and -C among KIR^- cells. The actually detected percentage of KIR^+ $NKG2\text{-}A^+$ or $\text{-}C^+$ cells divided by the calculated theoretical percentage without linkage gives an "overrepresentation-factor" 1 would indicate neither positive nor negative correlation. For NK cells, the obtained factors were 0.5, 0.7 and 0.9 for $NKG2A^+$ KIR^+ cells and 1.1, 1.2 and 1.3 for $NKG2C^+$ KIR^+ cells, indicating that co-expression might be negatively correlated concerning NKG2A and positively concerning NKG2C. In contrast, for T cells both NKG2 receptors were found positively correlated with KIR expression. $NKG2A^+$ KIR^+ cells were 2.2, 3.1 or 5.7 times and $NKG2C^+KIR^+$ cells 11.4, 12.4 or 14.4 times more frequent than they would be expected to occur if expression would be random within the T cell population.

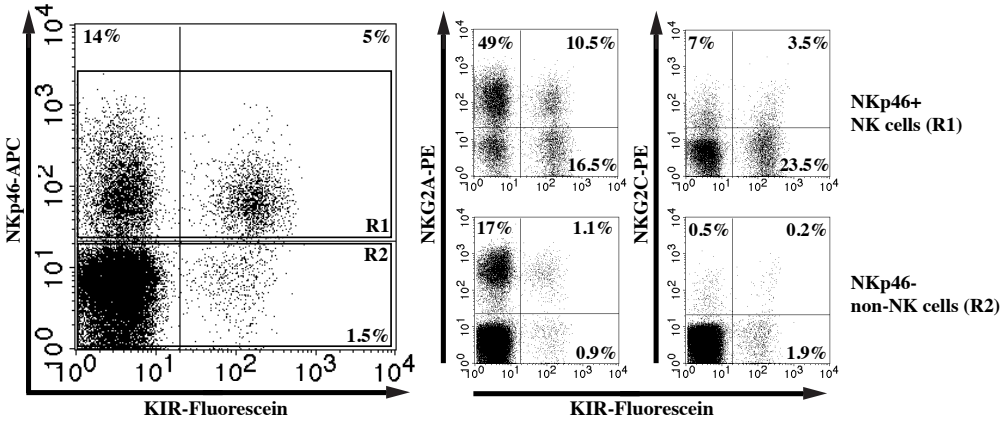

Figure 3.6 KIR expression on T and NK cells
Exemplary PBL staining using anti-NKG2-A (or -C), anti-NKp46 and anti-KIR (recognizing KIR2DL2, KIR2DL3, KIR2DS2 and KIR2DS4) antibodies. NK cells are identified as the major KIR expressing subpopulation. To determine the correlation between KIR and NKG2 receptor expression on T and NK cells, electronic gating on $NKp46^+$ NK cells (R1) and non-NK cells (R2) was used (see text for further details)

3.1.4 Amplification of CD94/NKG2 receptor-expressing cell types upon co-culture of PBMC with HLA-E expressing cells

To investigate the influence of HLA-E, the ligand for CD94/NKG2 receptors, on T and NK cell growth and/or survival and receptor expression, we co-cultured PBMC with the LCL 721.221 HLA class Ia-deficient lymphoma cell line (Shimizu et al., 1988) either transfected with HLA-E (221-AEH) or untransfected (221-wt) lacking HLA-E surface expression (Fig. 3.7). To monitor the total PBMC cell number at various time points after co-culture, representative samples were taken and counted in a haemocytometer. From this the cumulative cell number (CCN) was

calculated, which represents the amount of cells that would have accumulated without taking samples at a previous time-point (Fig. 3.8). Analyzing cells from six individuals, a general pattern of cell expansion was observed: PBMC co-cultured with 221-wt cells expanded until day seven and then declined again. This early expansion was missing in 221-AEH co-culture, which led to a more delayed but then stronger cell proliferation starting around day 7 and continuing for 5 to 7 days. The lifespan of the proliferated cells was short, as a rapid drop in cell numbers occurred afterwards. If PBMC were cultured alone, hardly any proliferation was detectable.

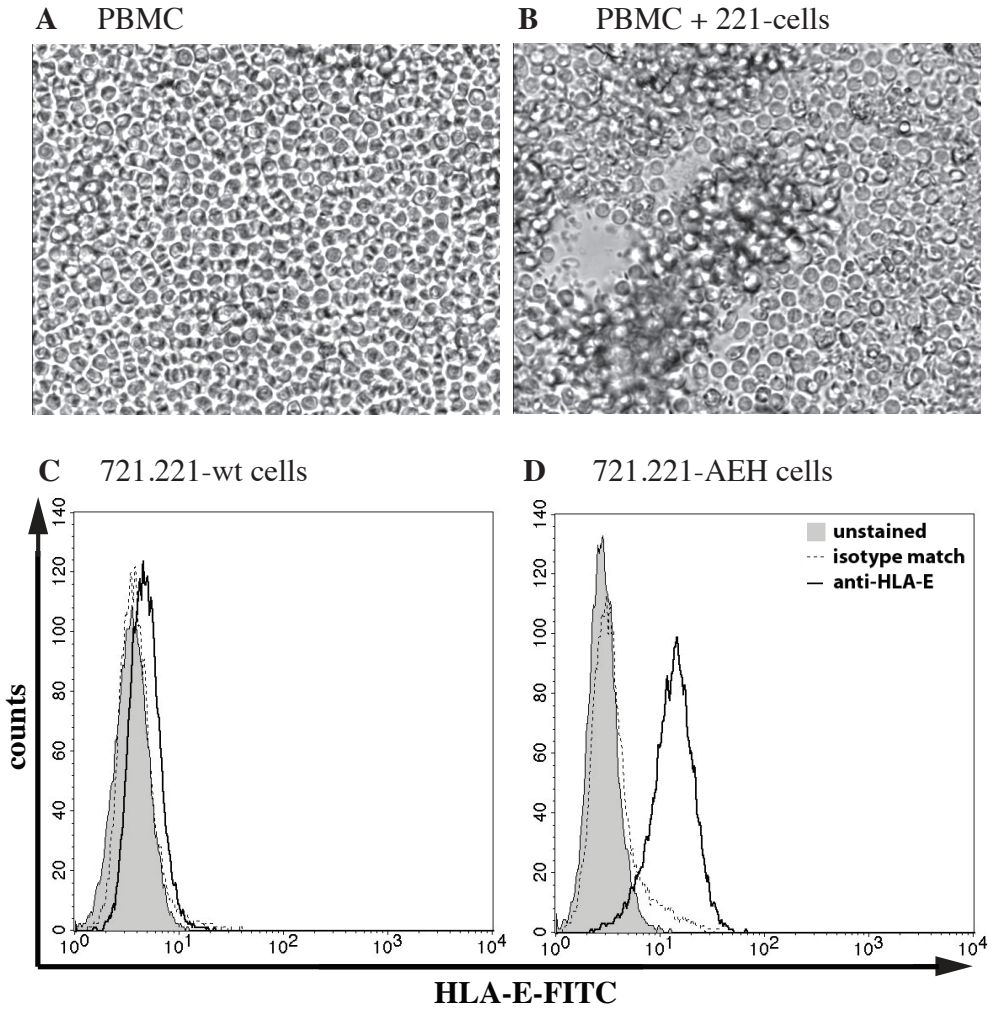

Figure 3.7 PBMC co-culture set-up
A, *B*, Freshly isolated PBMC alone (A) or mixed with irradiated 221 cells (B). *C*, *D*, confirmation of HLA-E expression on 221-AEH but not 221-wt cells. The primary unlabeled anti-HLA-E (MEM-E/08) antibody was detected with secondary anti-IgG-FITC.

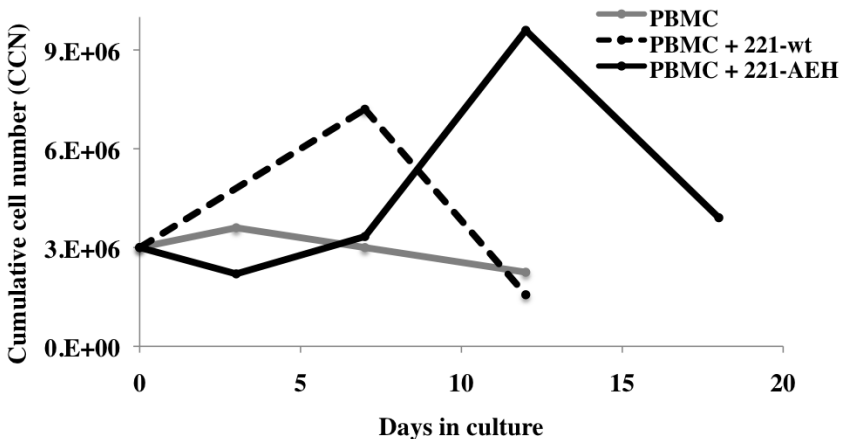

Figure 3.8 Growth curves of PBMC cultured alone or with 221 cells
Cells were counted at various time points using a haematocytometer. A representative donor for six analyzed is shown.

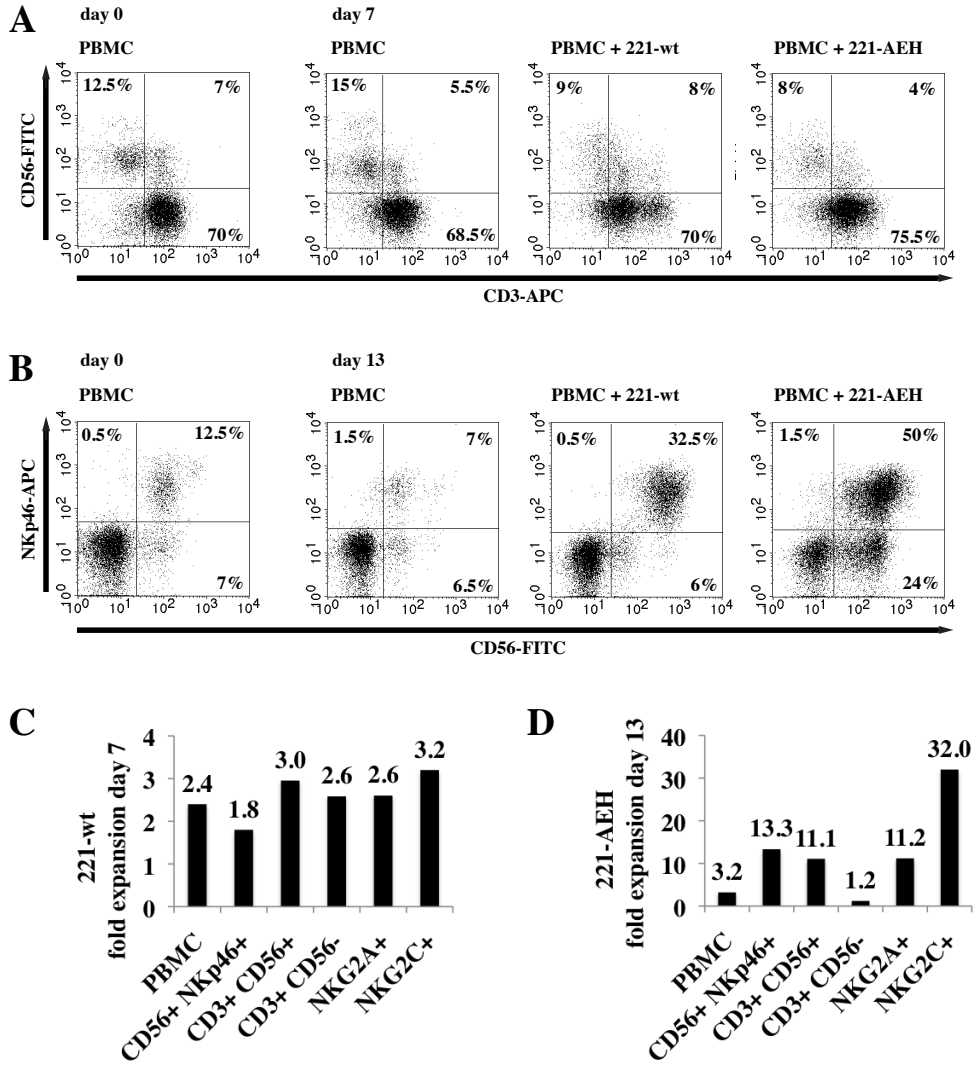

Figure 3.9 PBL composition and expansion upon 221-wt and 221-AEH co-culture
Isolated PBMC were co-cultured with 221-wt or 221-AEH cells and analyzed by flow cytometry. *A, B*, composition of PBL subpopulations at day 7 (A) and day 13 (B) upon various culture conditions. *C, D*, Multiplication of PBL subpopulations at the peak of the cell count upon 221-wt (C) or 221-AEH (D) co-culture relative to day 0. Representative for four experiments performed with one blood donor.

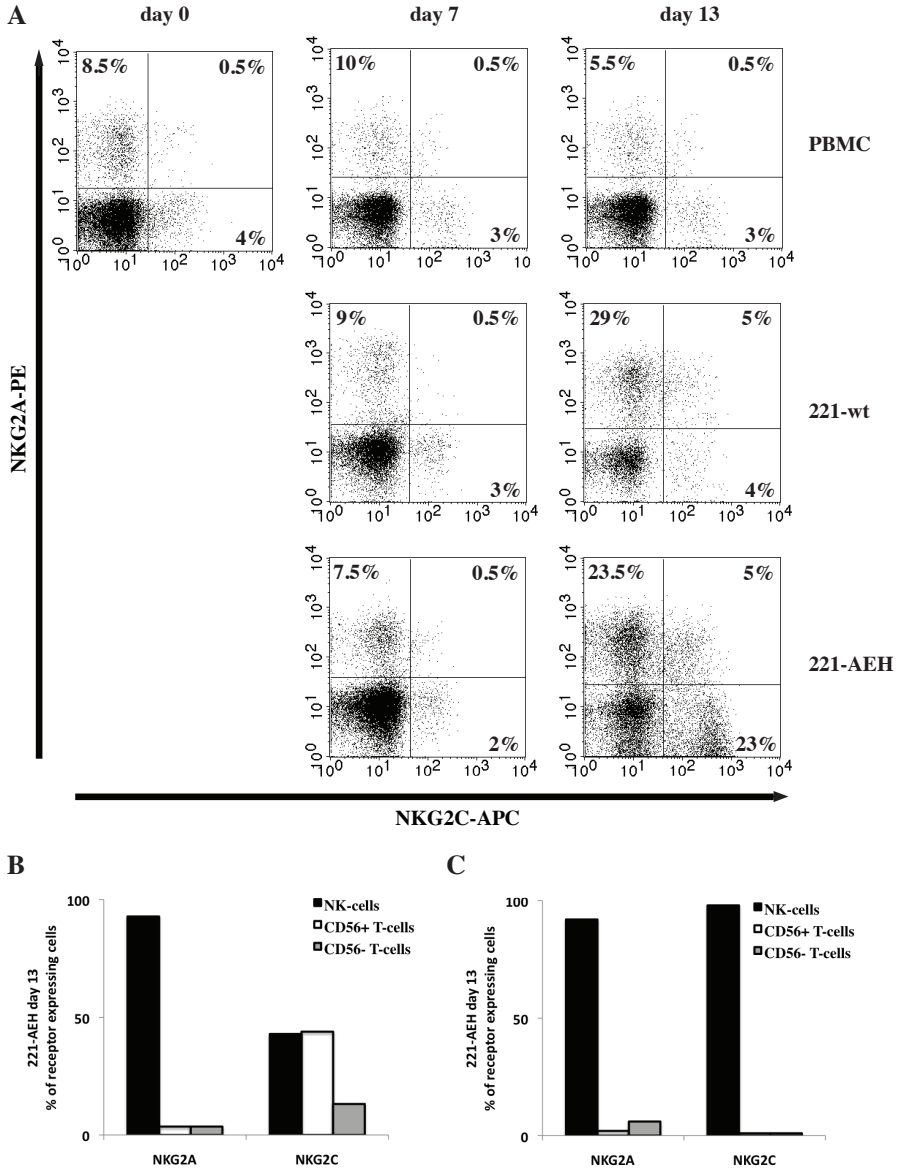

Figure 3.10 Expansion of NKG2C expressing cells upon 221-AEH co-culture
A, Representative graph for PBMC of six donors cultured alone, with 221-wt or 221-AEH cells and analyzed regarding the expression of NKG2A and NKG2C. *B*, *C*, Composition of NKG2A and NKG2C expressing cells upon 221-AEH co-culture for 13 days for PBL of donors showing both NK and T cell expansion (B) or only NK cell expansion (C).

To determine the nature of the proliferating cells, analysis by flow cytometry was performed. The peak at day 7 following 221-wt co-culture seems to be due to a general proliferation stimulus for all cells of the lymphocyte fraction and was comparable for all blood donors. (Fig.3.9 A & C) Whilst the amount of other cell types starts to decrease after day 7, NK cells seem to expand and/or survive until day 13 (Fig. 3.9 B).

A donor-dependent response was found for PBMC co-cultured with 221-AEH cells. Until day 7, no difference between the donors was observed, but the cell expansion between day 7 and day 13 differed. Principally, two extreme donor-types showing either $CD56^+$ T cell together with NK cell expansion (referred to as type 1 donors) or only NK cell expansion (type 2 donors) could be distinguished. Because an expansion of $CD56^+$ T cells was only detected upon co-culture with HLA-E expressing cells, and was not observed with 221-wt cells, we display the analysis focused on type 1 donors (Fig. 3.9 B). In line with previous reports by Guma et al., the most significant response takes place for NKG2C expressing cells (Fig. 3.9 D & 3.10) which were clearly preferentially expanded. Importantly, this response was found irrespective of the donor type indicating that both of the major CD94/NKG2 receptor expressing cell types, namely NK and $CD56^+$ T cells (see Fig. 3.5), can be induced to proliferate as a result of HLA-E encounter. For exemplary type 1 donors, amplified NKG2C expressing cells belong to an equal extent of about 43% to NK or $CD56^+$ T cells and about 13% can be detected in CD56 negative T cells. In contrast, NK cells constitute more than 90% of NKG2A expressing cells (Fig. 3.10 B) arguing for an inhibitory effect of NKG2A on T cell and in particular $CD56^+$ T cell proliferation as $CD56^+$ $NKG2A^+$ T cells outnumber $CD56^+$ $NKG2C^+$ T cells in freshly isolated PBMC (see Fig. 3.5). The strong correlation of CD56 and NKG2C expression on T cells is further emphasized by the finding that cells from type 2 donors (which do not respond to HLA-E expressing cells with $CD56^+$ T cell expansion) completely lack an expansion of NKG2C expressing T cells (Fig. 3.10 C).

3.2 Impact of CD94/NKG2 encounter of HLA-E on cell activation

3.2.1 Methodology (CD107a surface mobilization assay)

To evaluate the capacity of CD94/NKG2A and CD94/NKG2C to regulate cell activation (degranulation) upon encounter of target cells, CD107a surface mobilization assay was performed. Three-color flow cytometriy analysis with specific antibodies against CD107a, NKG2-A or -C, and NKp46 or CD3 allows to electronically gate on T and NK cells expressing NKG2-A^+ or -C^+ and subsequently analyze these cells for CD107a expression (Fig. 3.11 A). The amount of $CD107a^+$ NKG2-A and -C double negative NK or T cells was calculated by subtracting $NKG2A^+$ $CD107a^+$ and $NKG2C^+$ $CD107a^+$ from total $CD107a^+$. Freshly isolated PBMC contained a very low number of NKG2-A^+ -C^+ (referred to as double positive) cells which was found to be $\leq$ 0.5% of total PBL. Therefore, their double-representation within $NKG2A^+$ and

NKG2C$^+$ cells was ignored. This simplification was not applicable for NK cells when PBMC were co-cultured with 221 cells for 13 days prior to the CD107a surface mobilization assay because the amount of double positive cells sometimes even outnumbered NKG2C single positive cells after cell expansion. To overcome this problem, we negatively selected NK cells by magnetic-activated cell sorting (MACS) before incubation with target cells. The obtained high NK cell purity (> 98%) allowed then to analyze NKG2A$^+$, NKG2C$^+$, double negative and double positive NK cells without preceding electronic gating on NKp46$^+$ cells (Fig. 3.11 B). For this purpose, cells were triple-stained with antibodies recognizing CD107a, NKG2A or NKG2C. Degranulation-associated CD107a surface expression was investigated upon incubation with the prototypic NK target cells, the MHC class I deficient K562 cells (Britten, et al., 2002), as well as 221-wt and 221-AEH cells.

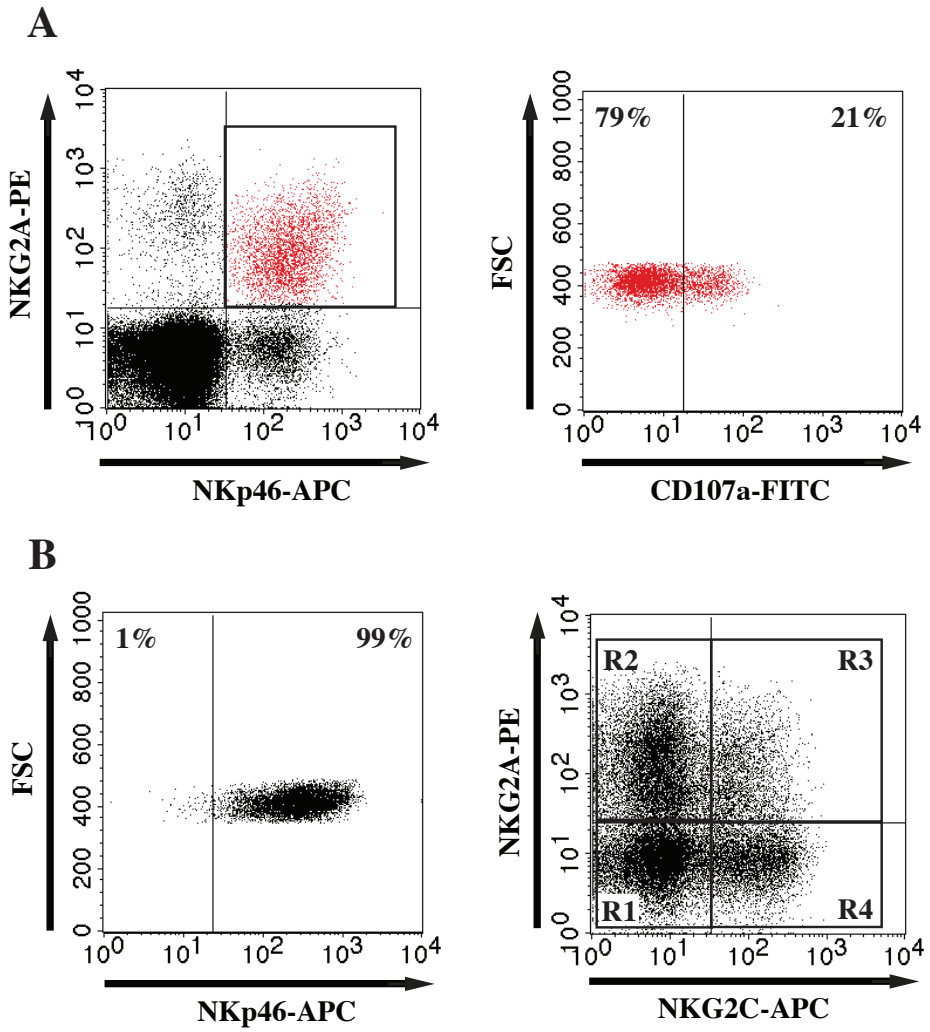

Figure 3.11 Gating strategy for CD107a surface mobilization assay
A, PBMC triple-stained with specific antibodies for CD107a-FITC, NKG2A-PE and NKp46-APC are gated on NKp46$^+$ NKG2A$^+$ cells (red) and subsequently analyzed for CD107a expression. To examine NKG2C$^+$ NK cells, NKG2C-PE is used instead of NKG2A-PE. The same strategy is employed for T cells by the use of CD3-APC instead of NKp46-APC. *B*, To be able to gate on NKG2-A and -C co-expressing NK cells, negative selection by MACS is used which gives high-purity NK cells (left plot). After incubation with target cells, triple-staining with NKG2A-PE, NKG2C-APC and CD107a-FITC antibodies allows to gate on NKG2-A and -C double negative (R1), NKG2A$^+$ (R2), NKG2-A$^+$ and -C$^+$ (R3) and NKG2C$^+$ (R4) NK cells. These gates are used for the analysis of CD107a as indicated in (A).

3.2.2 Reactivity of freshly isolated PBL

NK cells from freshly isolated PBL, expressing NKG2-A or -C show about the same reactivity towards K562 and 221-wt cells when tested in the CD107a degranulation assay. When compared to double negative NK cells, the NKG2 isoforms expressing Nk cells are the most reactive cells against MHC class I deficient targets. Encounter of HLA-E on 221-AEH cells clearly inhibits the activity of NKG2A$^+$ NK cells as compared to NKG2C$^+$ cells, which display high reactivity with all three target cells (Fig. 3.12 A).

Only a small fraction of T cells express CD94/NKG2 receptors and only this T cell fraction displays some CD107a on the surface. However, this is not found to be induced by target cell encounter since CD107a is found to a similar percentage on the surface of T cells incubated without targets (Fig. 3.12 B). Furthermore nodifference is found for NKG2A$^+$ and NKG2C$^+$ cells.

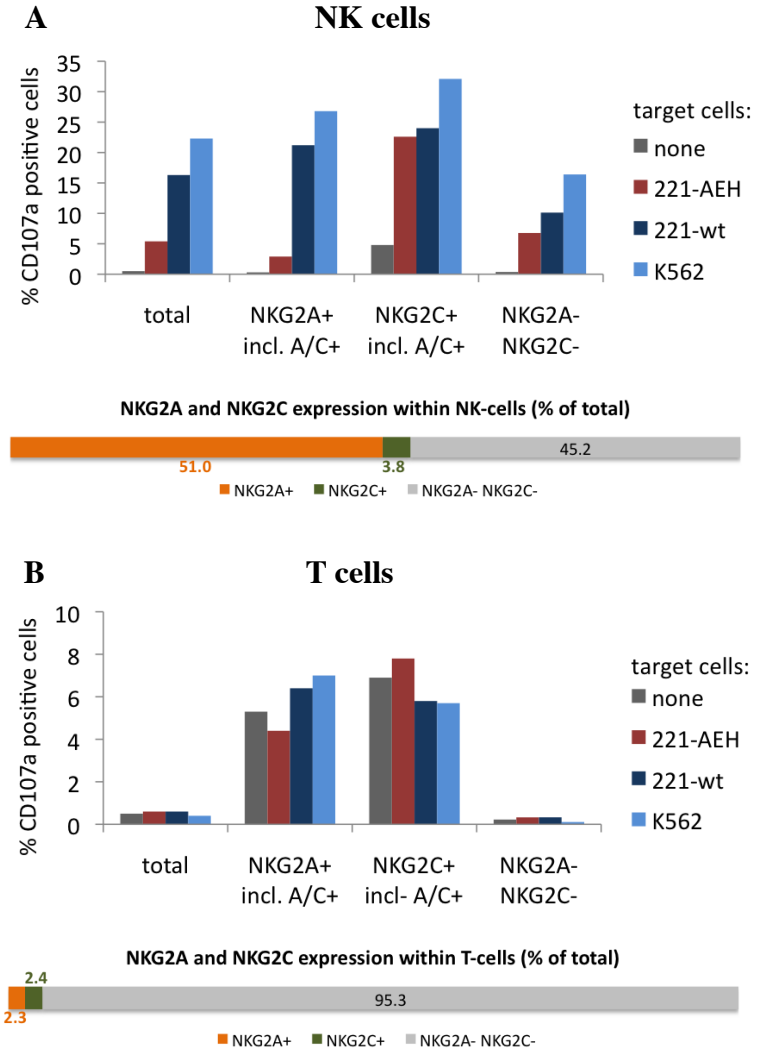

Figure 3.12 CD107a surface mobilization assay with freshly isolated PBMC
Total PBMC were incubated alone or with 221-wt, 221-AEH or K562 target cells for 5 hours. Thereafter, NK (A) and T cell (B) subpopulations were analyzed as described in figure 3.11. Cells expressing both NKG2A and NKG2C account for 0.2% of PBL. Values for double negative cells were calculated as described in 3.2.1.

3.2.3 Reactivity of PBL after co-culture with 221 cells

The expansion of NKG2C$^+$ cells upon long-term culture with HLA-E expressing target cells raises the question if these expanded cells would show altered reactivity against HLA-E cells. In this context, it is important to mention that 721.221 cells used for co-culture are lethally irradiated and are killed and/or disintegrated so that they are not detectable anymore by day 5. After 13 days of 221-AEH co-culture, the fraction of Nk cells inducible with any of the target cells investigated is reduced ba about 10% when compared to 221-wt co-cultured cells suggesting expanded cells have rather diminished activation potential. However, it was striking that after co-culture with 221-AEH as well as 221-wt the NKG2C$^+$ cells killed the HLA-e expressing 221-AEH cells significantly better than 221-wt cells suggesting that in this situation, HLA-E functions as triggering ligand. Surprisingly, in contrast to freshly isolated cells, co-cultured NKG2A$^+$ NK cells were hardly inhibited upon HLA-E encounter. The population of NK cells expressing both NKG2-A and -C showed an intermediate reactivity towards 221-AEH cells (Fig. 3.13). Taken, together, the results indicate that the progeny of cells induced to proliferate in the context of HLA-E does not differ from the cells cultured with 221-wt cells concerning their differential reactivity towards HLA-e target cells which is likely mediated via the CD94/NKG2 receptors.

We have further analyzed the CD94/NKG2 receptor expressing T cells, which werer largely CD56$^+$ and were amplified upon 221-AEH co-culture. These cells show similar strong activity when incubated with any of the target cells. Despite of a minor difference in reactivity against HLA-E negative targets, the NKG2 receptor isoform did not characterize functionally distinct subsets. As found for freshly isolated T cells, only the CD94/NKG2 receptor expressing population significantly expressed CD107a and again a sustantial portion displayed degranulation without addition of any target cell. Yet, long-term cultured cells show, unlike freshly isolated T cells, strong inducibility with NK target cells comparable to NK cells (Fig. 3.14).

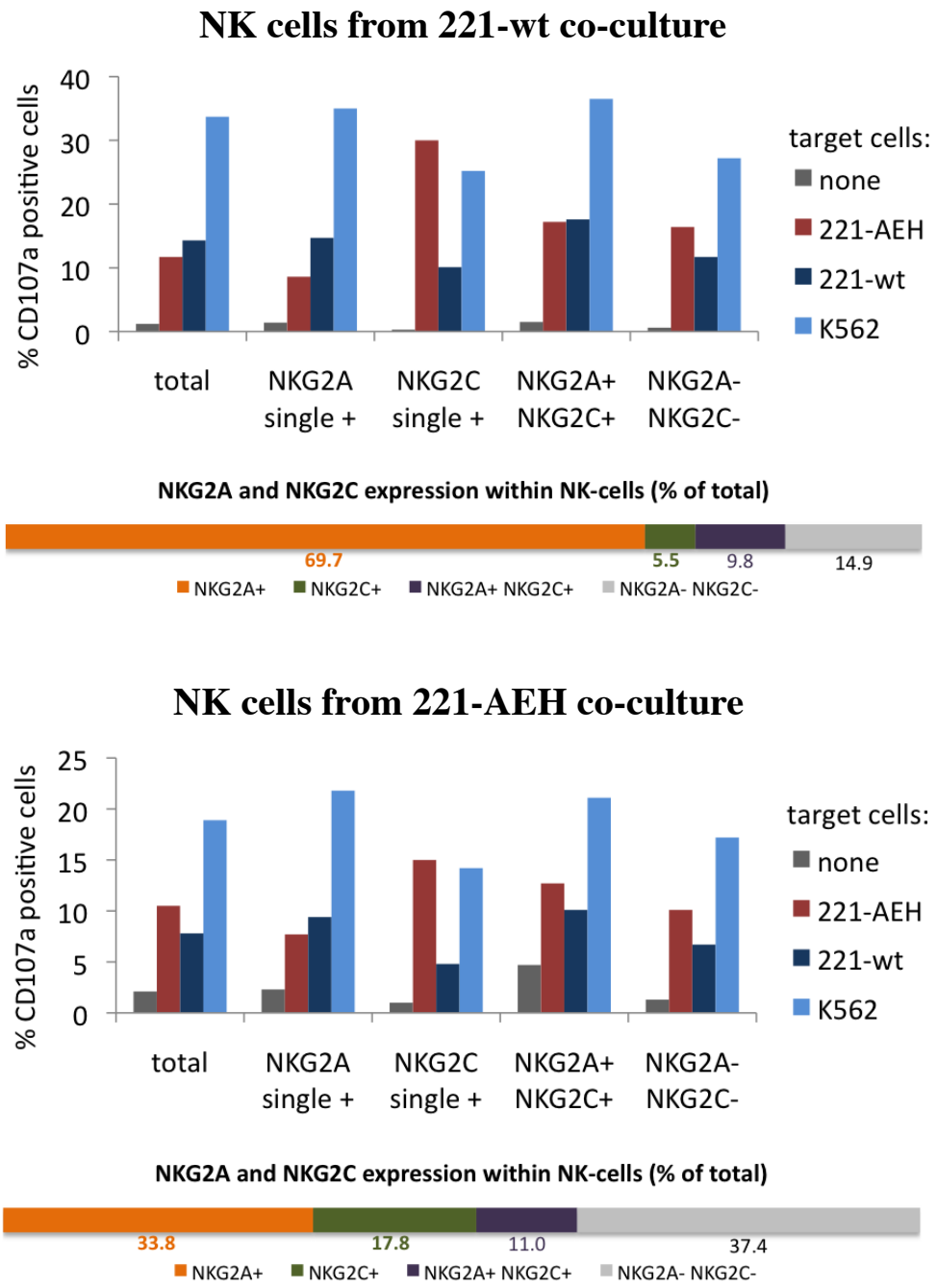

Figure 3.13 NK cell analysis of a CD107a surface mobilization assay after 13 days of PBMC co-culture with 221 cells

PBMC co-cultured for 13 days with 221-wt (upper graph) or 221-AEH (lower graph) were MACS sorted for NK cells. Isolated NK cells were incubated with target cells and analyzed as described in figure 3.11.

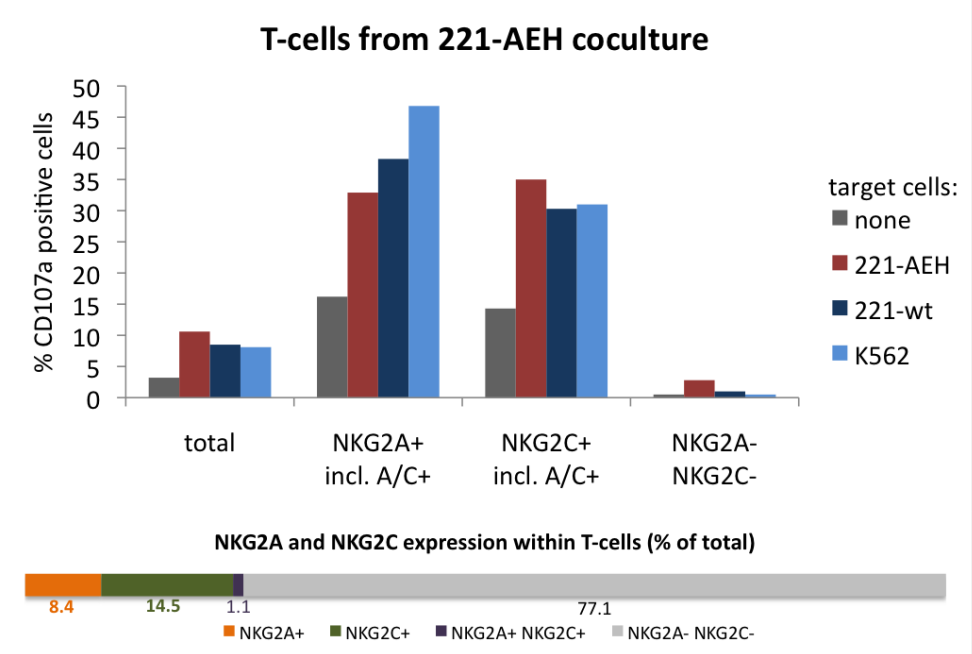

Figure 3.14 T cell analysis of a CD107a surface mobilization assay after 13 days of PBMC co-culture with 221 cells
Total PBMC were incubated with target cells and T cells were analyzed for CD107a surface expression as described in figure 3.11.

3.3. Responses to HCMV infected cells

Human cytomegalovirus (HCMV) infection is known to downregulate classical MHC class I whereas HLA-E expression on the surface of infected cells stays constant or is upregulated by specific immune evasion mechanisms (Tomasec, et al, 2000). Immune cells recognizing HLA-E via CD94/NKG2 receptors may therefore play a role in the immune reaction against HCMV. To investigate this possibility, we have studied co-culture of PBMC with embryonic lung fibroblasts (MRC-5 cells) infected with the AD169 HCMV lab strain.

3.3.1 Confirmation of HCMV infection and function

MRC-5 cells infected with AD169 show a multiplicity of infection (MOI) dependent cytopathogenic effect (Fig. 3.15 A). 18 hours post infection, fibroblasts become round, small but still adherent cells. This phenotype is reversible, lasts for two to three days until the typical fibroblast appearance is regained.
An indication for the functionality of HCMV in the *in vitro* system is the exertion of virus-specific modulation of cellular protein expression. The well-known downregulation of MHC class I genes (Hengel, et al., 1996) was used to confirm infectin 72 hours after HCMV addition (Fig. 3.15 B). At the same time, expression of CMV encoded FcR homologues was indirectly shown by increased binding of fluorescent labeled human IgG-Fc protein to infected cells as initially reported in 1976 by Keller et al. (Fig. 3.15 C).

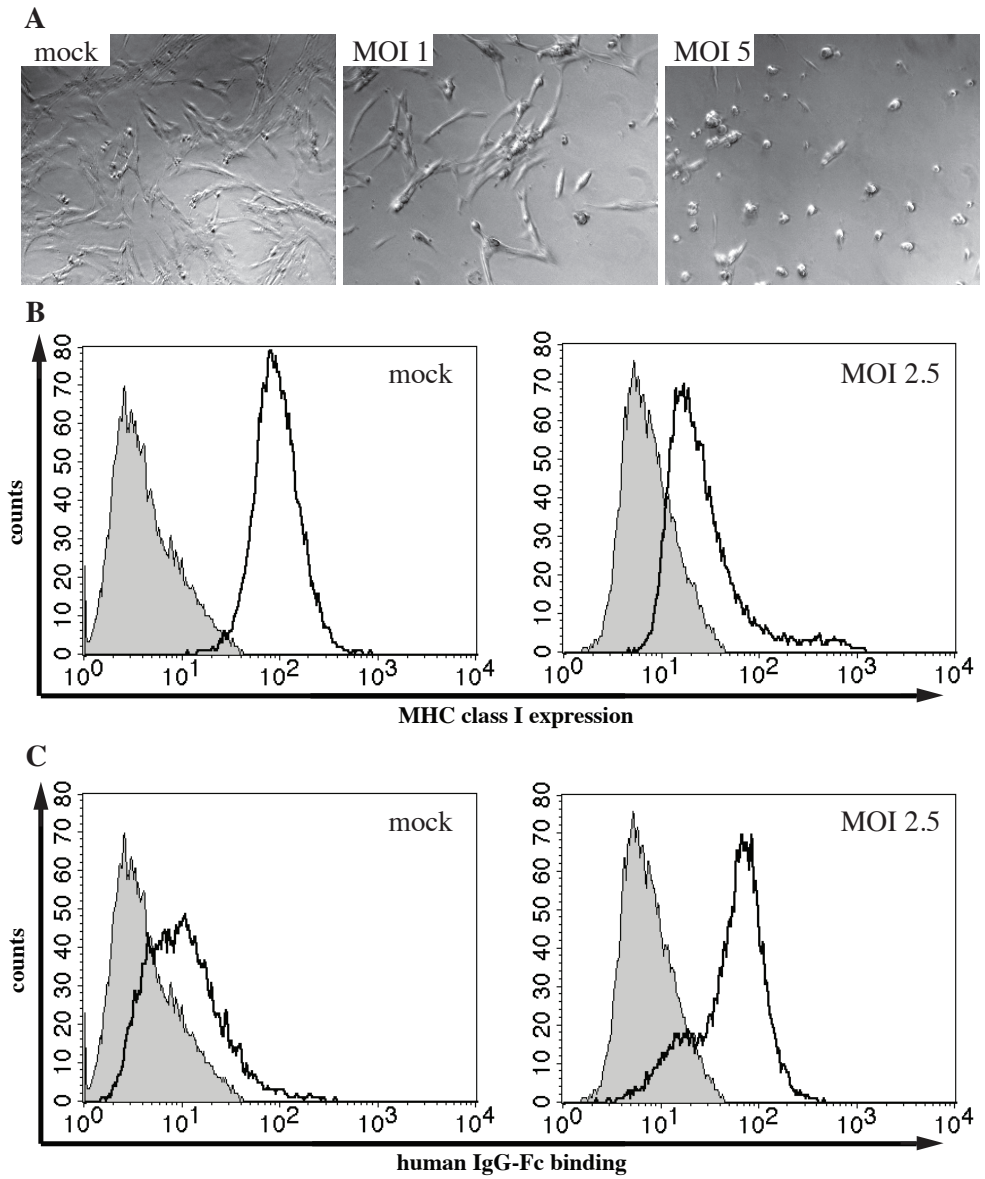

Figure 3.15 Conformation of CMV (Ad169) infection of MRC-5 cells
A, MRC-5 cells 18 hours post infection. *B*, *C*, Flow cytometry of MRC-5 cells 72 post infection. *B*, staining with a primary anti-MHC class I (W6/32) antibody detected with anti-IgG-FITC. *C*, cells were incubated with recombinant human IgG-Fc-FITC.

3.3.2 Differential responses of PBL upon exposure to HCMV infected fibroblasts

Co-culture experiments using PBMC of six different normal PBMC donors was performed including both CMV^+ and CMV^- individuals as tested by PCR and the presence of CMV specific antibodies in the serum. Aside of a generally reduced cell growth or survival upon exposure to CMV-infected cells, PBL from three donors (two of which CMV^- and one CMV^+) showed no significant change in NK- and T cell composition as well as expression of any other surface protein tested. For one of the two CMV^- donors, an expansion of NK cells was detected (**Fig. 3.16**).

Concerning CD56 expression, these NK cells were CD56^{bright} and many also CD16 positive. In line with the described preferential NKG2A^{+} phenotype of CD56^{bright} cells, 93.5% of these Nk cells expressed NKG2A. 21.5% expressed further NKG2C, indicating that a substantial portion of these the cells expressed both NKG2 receptors (Fig. 3.16 A & B). The NK cell expansion was found to be dependent on the MOI and was accompanied by a drop in T cells numbers. This is opposite of what is found when PBMC are cultured alone or with uninfected fibroblasts. In this case, NK cell numbers drop rapidly and T cells expand or survive preferentially (Fig. 3.16 C, left panels). Although the numbers of NK cells droped again after the expansion phase, at day 14 of co-culture with CMV infected fibroblasts, close to 80% of the lymphocytes of this donore were NK cells. On the other hand, T cells, which comprised 65% of freshly isolated lymphocytes were in this case found to be less than 20% after exposure to CMV (Fig. 3.16 C, right panels).

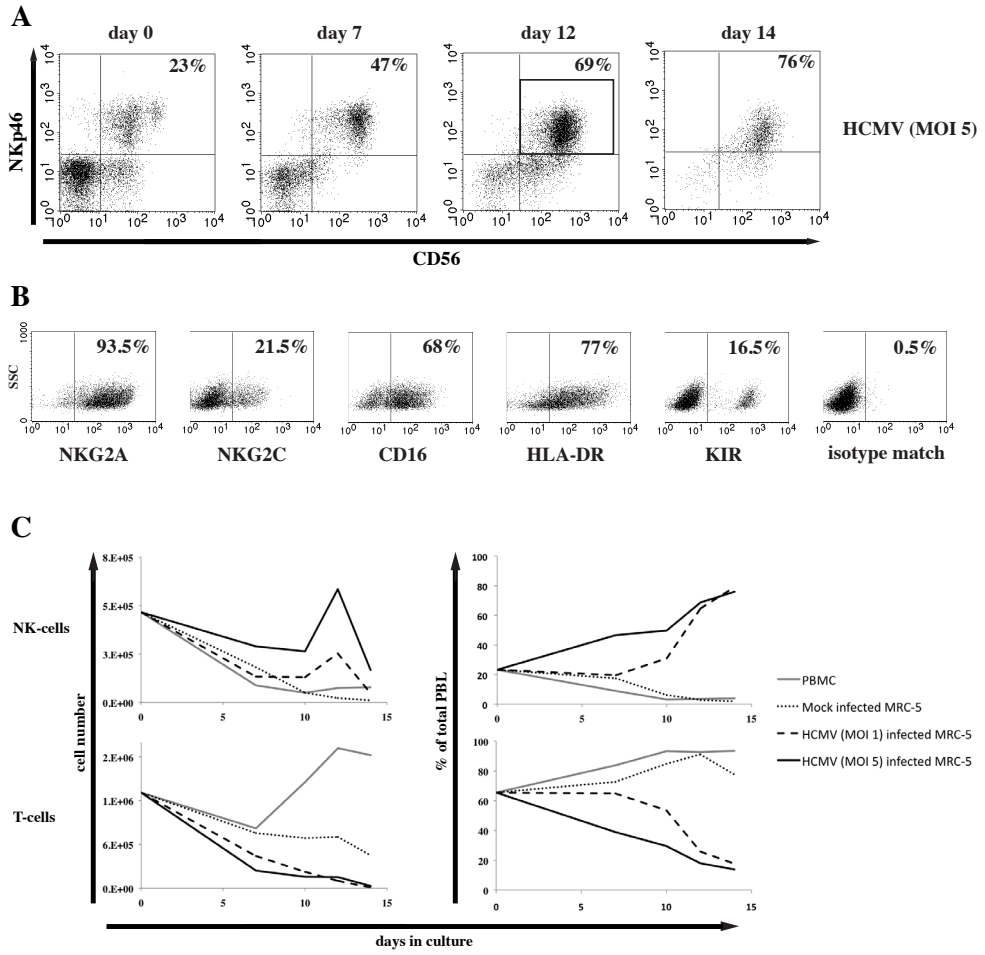

Figure 3.16 Expansion of NK-cells upon co-culture of PBMC with CMV infected fibroblasts

A, Flow cytometry of PBMC co-cultured with CMV (Ad169, MOI 5) or mock infected MRC-5 cells at day 7, 12 and 14. B, Receptor expression of NK cells at day 12 of co-culture. Gate used for analysis is indicated in (A). C, Cell numbers (left) of NK and T cells at various time points as well as their contribution to total PBL (right). Representative for two experiments of one CMV negative PBMC donor (number 5 in table 3.1).

For two donors (one CMV$^+$ by PCR, one CMV$^-$) a T cell response was obtained whereas other lymphocyte populations disappeared upon CMV exposure due to cell death. Infected fibroblasts (MOI 1) led to a larger proportion of T cells expressing CD56 (Fig. 3.17). In this case, a MOI dependency was not found as incubation with superinfected fibroblasts led to rapid cell death of T cells as well. Increased frequency of CD56$^+$ T cells could be due to upregulation of CD56 expression or expansion of a small subset of T cells already expressing CD56. The remaining T cells were long-lived, being still viable until day 15 to 17 of co-culture, outlasting cells co-cultured with mock infected fibroblasts. NKG2A expression increased within both CD56$^-$ and CD56$^+$ T cells until day 10 or day 12, respectively, followed by a subsequent decrease (Fig. 3.18). In contrast, NKG2C was hardly detectable on CD56$^-$ T cells throughout the co-culture but was expressed by CD56$^+$ T cells. After a slight drop of NKG2C expressing cells until day 12, the decreased NKG2A expression at day 15 goes along with a relative increase of NKG2C expression on CD56$^+$ T cells. Interestingly, the cell surface level of CD56 on NKG2C$^+$ T cells was comparable to CD56^{bright} NK cells.

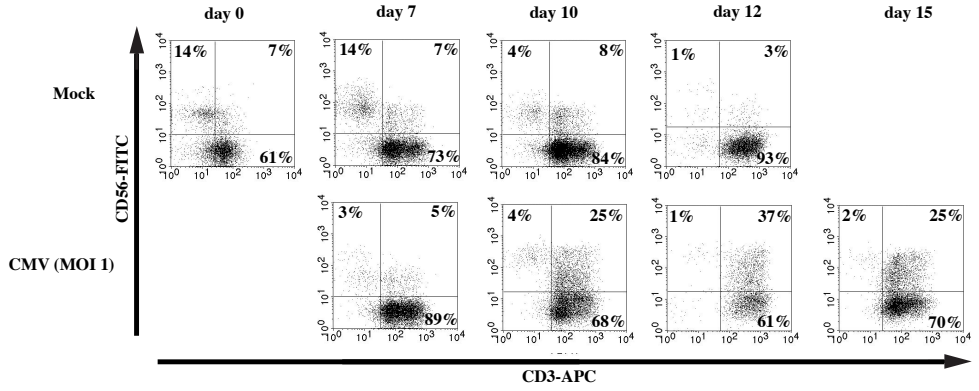

Figure 3.17 Increased expression of CD56$^+$ on T cells upon co-cultore with CMV infected fibroblasts

PBMC were co-cultured with CMV (Ad169, MOI 1) infected or mock infected MRC-5 cells and their surface protein expression was analyzed at day 7, 10, 12 and 15. Shown is an experiment with PBMC from donor 1 of table 3.1, representative of four performed with cells from two different donors.

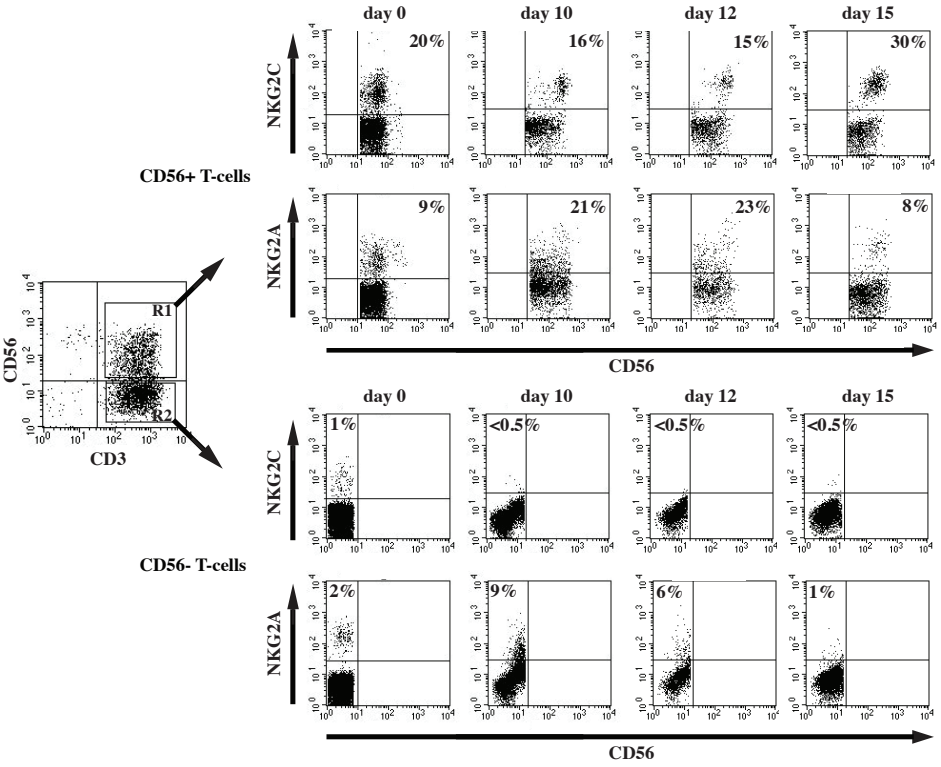

Figure 3.18 NKG2-A and -C expression on T cells upon PBMC co-culture with CMV infected fibroblasts

Analysis of PBMC co-cultured with CMV (Ad169, MOI 1) infected MRC-5 for the expression of NKG2A and NKG2C. Cells were triple-stained with anti-CD56-FITC, anit-CD3-APC and anti-NKG2A-PE or anti-NKG2C-PE. An exemplary dot-blot shows the regions used to gate on $CD56^+$ (R1) and $CD56^-$ (R2) T cells. Shown is an experiment representative for two performed with donor number 1 of table 3.1.

3.3.3 Preparation of a CMV (TB40) virus stock

The response of PBMC to virus infected cells may depend on both the virus strain and the cell type used for infection and co-culture. Ad169, the HCMV lab-strain used for all co-culture experiments, is not capable of infecting endothelial cells, a cell type infected by HCMV *in vivo* (Grefte, et al., 1993) and therefore of interest for the use in an *in vitro* co-culture system. Another HCMV lab-strain, called TB40, still carries the capability to infect endothelial cells such as Human Umbilical Vein Endothelial Cells (HUVEC). To prepare a TB40 stock for co-culture experiments, the virus was amplified in MRC-5 cells, purified using a sorbitol gradient, centrifuged, resuspended in PBS, aliquoted and titrated (see chapter 2.2 for more information). In total, 3 ml TB40 suspension with a titer of 5 x 10^7 plaque-forming units (PFU) per ml was obtained. 72 hours post infection of HUVEC with TB40, cells showed increased binding of human-IgG-FC and about two thirds of the cells downregulated MHC class I expression. The remaining one third upregulated MHC class I, probably representing cells where the HCMV life-cycle is not yet completed (Fig 3.19). Thus a TB40 stock for future studies in the context of endothelial CMV infection was successfully obtained and will allow the investigation of the interaction of NK and T cells with CMV infected endothelial cells.

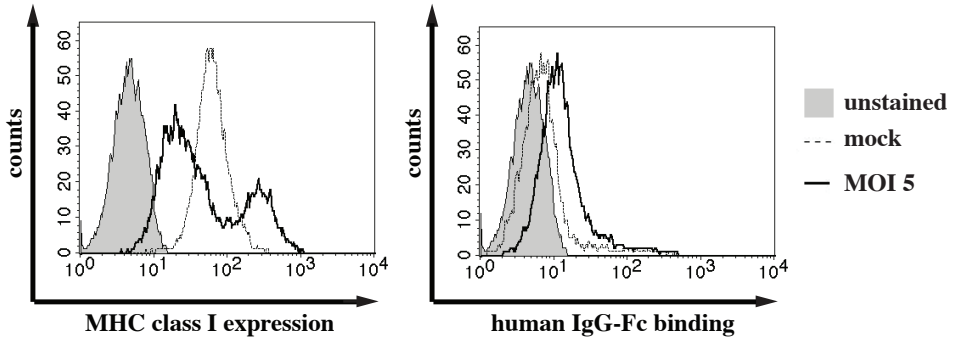

Figure 3.19 Confirmation of HUVEC infection by CMV (TB40)
Flow cytometry of HUVEC 72 hours post infection. Cells were incubated with a primary anti-MHC class I (W6/32) antibody and with anti-IgG-FITC for detection (left histogram) or with recombinant human IgG-Fc-FITC (left histogram). The fraction of HUVEC that up-regulated MHC class I surface expression could represent yet uninfected cells which have encountered CMV.

4. Discussion

In this work, we have investigated subpopulations of NK and T cells characterized by the expression of the CD94/NKG2 receptors and tested potential functional differences of these populations. This is of relevance since it became apparent over the last decade that certain subsets of peripheral blood lymphocytes, previously thought to constitute homogenous populations with rather common functions, have to be further subdivided to reflect the actual situation.
For example, expression of the Ig-superfamily member CD56 (N-CAM) was identified to be a useful surface marker on NK cells and a subset of T cells, although its function on these cells remains elusive as neither homophilic binding (as reported for neuronal cells) nor an impact on cytotoxicity was detected (Lanier, et al., 1991). Especially concerning natural killer cells, the expression pattern of CD56 was useful to distinguish between primarily cytotoxic cells being $CD56^{dim}$ and mainly cytokine producing $CD56^{bright}$ cells. The functional difference is indicated on the one hand by differential expression of CD16, a low affinity receptor for IgG mediating ADCC and on the other hand by L-selectin (CD62L) which is a homing marker for peripheral lymph nodes (figure 3.2). In human peripheral blood, the predominant NK cell subset, representing 90% of NK cells, are $CD56^{dim}$ $CD16^{+}$ $CD62L^{low/-}$ and the remainder is $CD56^{bright}$ $CD16^{low/-}$ $CD62L^{+}$. Conventionally, natural killer cells are identified in flow cytometry as $CD3^{-}$ $CD56^{+}$ or $NKp46^{+}$ cells. A small population of $NKp46^{+}$ $CD56^{-}$ NK cells is excluded if $CD3^{-}$ $CD56^{+}$ is used for identification, however these cells seem to have a dysfunctional phenotype, e.g. when analyzed in viremic HIV patients where they are expanded (Mavilio, et al., 2005). This indicates the importance of CD56 expression for the appropriate functioning of NK cells. Furthermore, CD56 is expressed on peripheral blood T cells, which comprise about 9% of the total $CD3^{+}$ population and are enriched in CD8 positive cells (> 90%; Ohkawa, et al., 2001). Within the $CD8^{+}$ population, CD56 expression was found to correlate with an effector phenotype (Pittet, et al., 2000). Cytokine-induced activation and potent anti-tumor functions similar to mouse $NK1.1^{+}$ T cells, termed NK-T, led to the use of this term for human $CD56^{+}$ T cells (Campbell, et al., 2001). However, it is important to distinguish NK-T from the CD1d restricted iNKT, which represent less than 1% of $CD3^{+}$ $CD56^{+}$ cells (Ohkawa, et al., 2001) and only a subset of iNKT expresses CD56 (Kim, et al., 2002).

4.1 CD94/NKG2 receptor expression in peripheral blood

In this work, members of the so-called “natural killer cell receptors” (because initially and mainly found on NK cells), the heterodimeric CD94/NKG2 receptors were analyzed to investigate their potential to characterize additional functionally distinct subpopulations of NK and T cells. NKG2 receptor chains are only expressed on the cell surface when paired with the invariant CD94 chain and 5 different NKG2 variants have been described to be generated from 3 distinct genes and by differential splicing. Of these the heterodimers of CD94/NKG2A or

CD94/NKG2C are the most prominently expressed forms, which makes theses two members the most promising candidates to exert important activities. Though both CD94/NKG2-A and -C recognize the same ligand, they transmit converse signals, raising the question if differential expression classifies functionally distinct PBL subsets. The expression of NKG2 receptors in non-activated PBL is to a large degree mutually exclusive, but a rare, consistently found population expresses both NKG2A and NKG2C (Fig. 3.3). Considering the antagonistic signals, a mechanism to regulate NK cell activation by means of transient NKG2 receptor co-expression was proposed. Furthermore, NKG2A was found to be upregulated on NKG2C^{+} cells following treatment with IL-12 whereupon these cells are inhibited for cytotoxicity against LCL 721.221 cells transfected with the corresponding ligand HLA-E (Sáez-Borderías, et al., 2009).

Analysis of NKG2-A and -C in CD56^{+} NK and T cell subpopulations revealed a preferential expression of both receptors on CD56^{bright} NK as compared to CD56^{dim} and an overrepresentation within the CD56^{+} T cell subset (Fig. 3.5). Because CD56^{bright} NK and CD56^{+} T cells account for only 1.5% and 6% of PBL, whereas CD56^{dim} NK and CD56^{-} T cells represent about 15% and 64%, respectively, the majority of NKG2-A and/or -C expressing cells belong to the latter groups. In either case, the surface expression of the inhibitory NKG2A isoform clearly outnumbers all other CD94/NKG2 receptors as most of the cells that stain for the invariant CD94 chain also stain for NKG2A (Fig. 1.2 A). This argues for an overriding importance of NK and probably also T cell inhibition via CD94/NKG2A in healthy individuals. Furthermore, the expression of the inhibitory NKG2A on NK cells scarcely differed between individuals, whereas the activating NKG2C varied widely. Deletion of NKG2C within healthy individuals (Miyashita, et al., 2004) and joint transcriptional regulation of both isoforms in NK cells (I. Karas, Ph.D. thesis, 2009), provides evidence for a similar function of the activating isoforms. Aside of that, co-expression of NKG2C and NKG2E may occur and cooperation would likely promote NK cell activation upon HLA-E encounter. However, the lack of a specific antibody for NKG2E did not allow to investigate this assumption.

In regards of the diverse group of killer immunoglobin-like receptors (KIR) which recognize various MHC class I molecules and comprise, similar to CD94/NKG2 proteins, inhibitory and activating members, these are, similar to the NKG2 receptors, mainly found on NK cells but also present on a small population of T cells. Usage of connatural or identical signaling motifs (mainly ITIMs and ITAMs) and recognition of "self" via MHC class I by both, the NKG2 and KIR receptor families, opens the possibility for partially overlapping functions. More than 50% of KIR expressing T cells are found to express in addition a CD94/NKG2 member (Fig. 3.6). Since on average, only about 2% of T cells express KIR and 6% express NKG2 receptors this indicates the existence of a specialized subset of T cells capable to express NKG2, KIR or both receptors. CD1d restricted iNKT cells are known to express various NCR but as they make up only 0.1% of PBL (Lee, et al., 2002), also canonical T cells, expressing a variable TCR, should therefore contain further specialized subpopulations characterized by NK receptors. They likely belong to the so-called NKT-like cells, usually classified by the expression of another NK re-

ceptor, CD161 (NKRP1), which exhibits a broad spectrum of functions (Godfrey, et al., 2004).

4.2 Expansion of PBL subpopulations upon 221 cell co-culture

Recognition of a specific ligand by a cell surface receptor could lead to changes in receptor expression as well as result in functional activation and/or proliferation of the cells. Some evidence has been reported that this may be the case for the encounter of HLA-E by CD94/NKG2C, as co-culture of PBMC with cells expressing HLA-E led to proliferation of $NKG2C^+$ cells (Guma, et al., 2005). We have therefore tested this in long-term in vitro culture of lymphocytes which can be supported by the addition of irradiated lymphoid cell lines (LCL) as feeder cells (Hardy, et al., 1970). To avoid allogeneic responses, we used the MHC-class Ia deficient LCL 721.221 (221-wt) cell line. This mutant of the B cell lymphoblastoid cell line LCL.721 (Shimizu, et al., 1988), which was generated by γ-ray mutagenesis, expresses intracellular but not cell-surface HLA-E because MHC-class I derived peptides are needed for surface expression of HLA-E and these cells lack classical MHC class I (Lee, et al., 1998). To investigate the influence of HLA-E, the ligand for NKG2A and -C, LCL 721.221 cells transfected with an expression construct for HLA-E, termed 221-AEH, were used. The HLA-E expression construct contains a hybrid HLA-E gene in which the HLA-E leader peptide sequence was substituted for the HLA-A2 signal peptide sequence. The HLA-A2 signal peptide generated loads into the HLA-E peptide binding site and leads to cell-surface expression of bona-fide HLA-E by 221-AEH cells (Lee, et al., 1998).

In the established assay, PBMC were co-cultured with 221-wt or 221-AEH cells and cell numbers as well as surface receptor expression was monitored over a period of two weeks. At a first glance, an obvious difference in the time frame of cell expansion was observed in dependence of the cells added to the PBMC. PBMC cultured with 221-wt cells showed a peak of cell expansion at day 7 and those cultured with 221-AEH cells at day 13 whereas culture without 221 cells led to no real expansion during the whole period (Fig. 3.8). Within the lymphocytic fraction, T cells accounted for the cell expansion upon 221-wt co-culture. This expansion of T cells was found to a significantly lesser degree in 221-AEH co-cultures (Fig. 3.9 A). The observed T cell expansion is likely driven by a cytokine/chemokine milieu produced and cell-cell contacts provided by the irradiated feeder cells. It remains however unclear why 221-wt cells have stronger effects in this respect when compared to 221-AEH cells. In contrast to T cells, NK cells started to proliferate only after day 7 of either co-culture and displayed, in dependence of certain donors, a more strongly pronounced amplification upon 221-AEH co-culture peaking at days 11-13 (Fig. 3.9 B). The expanding cells had the appearance and the surface markers of large granular lymphocytes and seemed rather short-lived as a rapid drop in cell numbers occurred following cell expansion. Interestingly, presence of HLA-E on the cells in the co-culture system led to a preferential outgrowth of NK and T cells expressing the activating CD94/NKG2C receptor (Fig. 3.10 A). It seems that triggering of the activating CD94/NKG2C receptor by HLA-

E can either upregulate NKG2C expression and/or give a preferential growth signal to NKG2C expressing cells. However, since cells expressing NKG2A also amplified, although usually not to the same extent, the events in the culture are more complex. It could be that NKG2C cells stimulated by HLA-E would secrete growth factors providing a stimulus also to the NKG2A expressing cells or NKG2A expression on immature cells in the mixture can be directly triggered by HLA-E or other growth promoting molecules of the feeder cells. Another effect mediated preferentially by the 221-AEH cells, the appearance of $CD56^+$ T cells with NKG2C expression was not found for all PBMC donors. Importantly, NKG2C in the amplified cultures was expressed by both NK and T cells, when the latter were also amplified. NKG2C expression was mostly on $CD56^+$ T cells (Fig. 3.10 B & C). In contrast, $NKG2A^+$ cells were expressed almost exclusively on NK cells. In this regard, it is important to note that NKG2A positive cells outnumber NKG2C cells by far when freshly isolated T cells are analyzed, i.e. when the final culture contained comparable amounts of NKG2A and NKG2C cells, the NKG2C cells had much more proliferated. Alternatively, we can not exclude that NKG2C expression was also upregulated on NK cells not expressing this receptor at the beginning of the culture.
It has been described for murine T cells expressing low levels of CD94 that they bind to tetramers of the HLA-E homologue Qa1 supported by CD8 whereas NK cells bound the tetramers only if high levels of CD94/NKG2 receptors were expressed (Gunturi, et al., 2003). If this is also true for humans, CD8 could be involved during the initiation of $NKG2C^+$ T cell expansion.

CD94/NKG2A expression was shown to be a clonal feature of a restricted subset of $CD8^+$ T cells, the main NCR expressing T cells, where TCR specificity is involved in the control of CD94/NKG2A expression and committed cells express cell-surface NKG2A following recent antigen encounter via TCR (Jabri, et al., 2002; Braud, et al., 2003). The MHC class-Ia deficiency of 221 cells may therefore explain that almost no $NKG2A^+$ T cells are generated upon co-culture as TCR stimulation via MHC class Ia can not occur. 221-AEH cells are modified to express the HLA-E*0101 allele, one of two variants frequently found in humans, and provide HLA-A2 signal peptides for loading into HLA-E. Considering that TCR specificity may also play an important role, donor dependency of the $NKG2C^+$ T cell response could be explained by absence or presence of allo-reactive TCR to the HLA-E allele and also the bound peptide can either be recognized as "self" ($HLA\text{-}A2^+$ donor) or "non-self" ($HLA\text{-}A2^-$ donor).
NKG2C mRNA is present in many $CD8^+$ T cells but CD94/NKG2C surface expression was reported to occur late in T cell development exclusively by highly differentiated effector cells (Arlettaz, et al., 2004). When murine cells were cultured *in vitro*, apoptosis was inversely related to the expression of CD94 on $CD8^+$ T and NK cells (Gunturi, et al., 2003), which could provide one explanation for the increased appearance of $CD94^+$ $NKG2C^+$ cells upon long-term co-culture. As NKG2C functions as a co-stimulatory receptor on T cells, it could play an important role during the final steps of an immune response. When the obtained TCR stimulation is insufficient due to low concentrations of the specific antigen, NKG2C might add to the activa-

tion. Similarly, the expansion of cells expressing NKG2C upon 221-AEH co-culture could be preferentially induced by the additional activating signal transmitted via NKG2C. Though NKG2A and NKG2C mRNA is present in some T cell clones, surface expression has been reported to be mutually exclusive (Arlettaz, et al., 2004). However, in this work we observed that about 1% T cells express NKG2A and NKG2C after 221-AEH co-culture (Fig. 3.14). These cells can be expected to be very rare among freshly isolated PBL and may amplify during co-culture.
HLA-E specific invariant $CD8^+$ T cells have been reported upon *Salmonella enterica* (Salerno-Goncalves et al., 2004) and *M. tuberculosis* (Heinzel, et al., 2002) infection and their prevalence in peripheral blood was recently reported (Aziz, et al., 2009). Furthermore, increasing evidence suggests that bacterial peptides, presented by HLA-E may lead to binding of corresponding TCRs (Heinzel, et al., 2002; Salerno-Goncalves, et al., 2004). However, as 221-AEH cells express HLA-E and an HLA-A derived peptide which should load into the HLA-E, it is more likely that T cells amplify in the co-culture due to NKG2C functioning as a co-receptor for T cell activation and not due to HLA-E recognition by TCR. The cell-surface level of HLA-E on 221-AEH cells is much higher as compared to 221 cells which can be induced to express HLA-E by the addition of peptides (Salerno-Goncalves, et al., 2004). This could promote the lower-affinity interaction of NKG2C and HLA-E, thereby inducing preferential $NKG2C^+$ cell expansion. NKG2C expression on T cells might be important to provide co-activation for example in the case of viral infections, as viruses can upregulate HLA-E surface expression (Tomasec, et al., 2000; Megret, et al., 2007).
Similarly, the (in comparison to T cells less pronounced) overrepresentation of $NKG2C^+$ NK cells when exposed to HLA-E expressing 221 cells could reflect a response mechanisms to abnormally high HLA-E surface expression.
Taking to account that most NK cell receptors were found to require co-stimulation of more than one activating receptor in order to induce activation (Bryceson, et al., 2006), CD94/NKG2C encounter of HLA-E could provide one of the important signals needed to activate NK cells, in addition signals from e.g. members of the leukocyte Ig-like receptors (LILR), which can bind conserved regions of MHC class I molecules might contribute.

4.3 Activation of CD94/NKG2 receptor-expressing cells

At first glance, the existence of inhibitory and activating receptors for the same specific ligand, sometimes even co-expressed on the surface of a single cell, is somewhat puzzling and might reflect tightly controlled regulatory mechanisms. Aside of CD94/NKG2-A and -C, this principle is also observed for killer-cell immunoglobulin-like receptors (KIR). For example, the inhibitory KIR2DL1 and activating KIR2DS1, both recognizing HLA-C, constitute oppositely signaling receptors recognizing the same ligand (Stewart, et al., 2005). Affinity differences of the receptor-ligand binding can favor the functional impact of the higher-affinity member. For receptors recognizing constitutively expressed “self” ligands like MHC class I molecules, the

inhibitory receptor form would essentially have to show higher affinity as compared to its activating counterpart to render such a mechanism possible without causing autoreactivity. The remarkable finding for KIR2DL1 and KIR2DS1 as well as CD94/NKG2A and CD94/NKG2C is, that this prerequisite is fulfilled. It points at its functional importance to control cell activation. On the other hand, CD94/NKG2E, another activating HLA-E specific receptor, was shown to have indistinguishable ligand binding affinities from CD94/NKG2A (Kaiser, et al., 2005). Unfortunately, to date no NKG2E specific antibody exists and the consequence of this can not be further analyzed. Because co-transcription of various NKG2 family members does not correlate with cell-surface protein co-expression (Arlettaz, et al., 2004), it would first be necessary to clarify wether NKG2E translation and surface mobilization takes place. Regulation of the sensitivity of cells towards activation via relative surface-levels of activating and inhibitory receptors for the same ligand would also be conceivable. The recently published IL-12 dependent upregulation of NKG2A expression on NKG2C$^+$ NK cells (Sáez-Borderías, et al., 2009) substantiates this possibility.

To address the impact of NKG2 receptor expression before and after co-culture on NK and T cell activation, CD107a surface expression was analyzed after incubation with MHC class I deficient target cell lines (221-wt and K562) and the HLA-E expressing 221-AEH cell line. CD107a (LAMP-1) is found ubiquitously on the luminal side of lysosomal and endosomal membranes and reaches the cell surface upon vessicle-membrane fusion and effector-molecule release. CD107a surface expression therefore correlates with cytokine secretion and cytotoxicity mediated by NK as well as CD8$^+$ T cells (Betts, et al., 2003; Alter, et al., 2004). Analyzing CD107a surface expression by flow cytometry is a possibility to assess the ability of these cells to degranulate, which serves as an indication for cell activity. Reflecting the eponymous property of NK cells to spontaneously kill MHC class I deficient targets, incubation of freshly isolated PBMC with 221-wt and K562 cells leads to activation of NKG2-A and/or -C expressing and non-expressing NK cells (Fig. 3.12 A). If 221-AEH cells were used as targets, NKG2A$^+$ NK cell activation was inhibited to a large extent, emphasizing the functional consequence of CD94/NKG2A receptor interaction with HLA-E. It has to be pointed out that this is an HLA-E dependent inhibition under otherwise activating conditions (HLA class Ia deficient target cells), indicating that HLA-E binding to NKG2A is a bona fide inhibitory “self” signal to NKG2A$^+$ NK cells. Unexpectedly, NKG2C$^+$ NK showed equal activity upon incubation with 221-wt or 221-AEH cells. This indicates an insufficient contribution of NKG2C to induce degranulation of NK cells activated due to missing classical MHC class I expression by the target cells.
In the case of T cells, NKG2-A and/or -C expressing cells make up only a very small fraction. CD107a surface expression was not increased by target cell incubation but around 6% of T cells expressing either NKG2 receptor displayed surface CD107a without encounter of target cells, which is in contrast to T cells not expressing NKG2 (Fig. 3.12 B). This spontaneous degranulation without target cells probably indicates an increased activity of NKG2 receptor expressing

T cells.

The next aspect to analyze was if long-term co-culture of PBMC with 221-wt or 221-AEH cells alters the reactivity towards target cells. Increased numbers of NKG2-A and -C co-expressing NK cells in the amplified population allowed to include this population in addition to cells expressing only NKG2A or NKG2C. The NK cells from co-culture with 221-wt and 221-AEH cells displayed largely similar differences in degranulation activity between the subpopulations defined by NKG2 receptor expression. This indicates that cells which specifically expand upon encounter of HLA-E do not change their functional properties in comparison to cells which do not expand to the same degree but are kept in co-culture with the feeder 221-wt cells. It turned out that one major difference compared to freshly isolated cells exists: $NKG2C^+$ NK represent the least activated population if MHC class I deficient target cells are used but get highly activated upon encounter of HLA-E suggesting that HLA-E can function as activating ligand for $NKG2C^+$ cells from the co-cultures. In parallel, the inhibitory effect of NKG2A interaction with HLA-E is reduced. It is conceivable that *in vivo*, upon infection with pathogens that may lead to upregulation of HLA-E surface expression by loading of viral peptides into HLA-E, increased numbers of NK cells expressing NKG2C and reactive with e.g. HLA-E/viral peptides would be beneficial and this could be further supported when at the same time the inhibitory effect of NKG2A after binding to HLA-E is less pronounced.

It has to be mentioned that NKG2A and NKG2C double-negative NK cells appeared to be more activated by 221-AEH cells than by 221-wt, suggesting a significant contribution of other receptors recognizing HLA-E. Potential candidates would be activating LILR and CD94/NKG2E or H.

The six fold higher affinity of CD94/NKG2A interaction with HLA-E as compared to CD94/NKG2C adumbrates that inhibitory signaling should dominate in NKG2-A and -C double positive cells. Surprisingly, the double-positive cells display intermediate activity and hardly any difference between 221-wt and 221-AEH target cell induced degranulation. It therefore appears that the very fast association and dissociation constant of the interaction between CD94/NKG2 and HLA-E (Vales-Gomez, et al., 1999) could probably make the affinity difference less relevant.

The expanding T cells expressing NKG2-A and/or -C upon 221-AEH co-culture, which are mainly $CD56^+$, display high levels of spontaneous CD107a surface expression without target-cell contact, similar to freshly isolated T cells. Hoever, T cells amplified after co-culture and expressing any of the two analyzed CD94/NKG2 isoforms can be induced to degranulate by MHC class I deficient target cells and this inducibility is a property largely restricted to NKG2-A and/or -C expressing T cells (Fig. 3.14).

Notwithstanding HLA-E seemed to induce the amplification of these cells, the response against 221-AEH cells does not significantly differ from other target cells. This again stresses the importance of additional activating interactions between the T cells and target cells. The small fraction of NKG2-A and - C negative T cell, activated upon 221-AEH cell co-culture, could be

comprised of cells recognizing HLA-E via TCR although further investigation is necessary to substantiate this assertion. Collectively, these results provide evidence that expression of CD94/NKG2 receptors by T cells can be used as a cell-surface marker to identify cells capable of TCR independent recognition of target cells.
Considering that HLA-E does not influence the inducibility, it is reasonable to hypothesize that additional ligands/receptors exist and account for the high reactivity of these cells. It is possible that the major function exerted by CD94/NKG2 receptors on T cells could be to facilitate the attachment to target cells more than a contribution to cell activation as degranulation is found largely independent of the CD94/NKG2 isoform.

4.4 PBL co-culture with HCMV infected fibroblasts

The importance of NK cells and cytotoxic T lymphocytes in viral defense has been clearly shown by a multitude of studies (Andoniou, et al., 2006; Wiesel, et al., 2009). Human cytomegalovirus (HCMV) uses various strategies to evade host immune reactions enabling the virus to establish latent infection. Amongst them are mechanisms that specifically target MHC class I expression, including downregulation of classical MHC class Ia whilst keeping or increasing HLA-E expression (Tomasec, et al., 2000). Guma et al. (2004) provided evidence that CMV can shape the NK and T cell receptor repertoire in healthy carrier individuals, resulting in increased frequencies of $NKG2C^+$ cells. Furthermore, $NKG2C^+$ NK cells were found to be specifically amplified upon co-culture of PBMC with CMV infected embryonic lung fibroblasts (MRC-5 cells) (Guma, et al., 2006). Absence of the viral gene region US2-11, responsible for MHC class Ia downregulation, impaired the response whereas MHC class I homologues and HLA-E upregulating UL40 did not have an effect. MRC-5 cells reportedly do not express cell-surface HLA-E (Tomasec, et al., 2000; Guma, et al., 2006), suggesting either an indirect effect leading to $NKG2C^+$ NK cell amplification or the existence of a yet undefined ligand of host or viral origin active as amplification stimulus. As described above, co-culture of PBMC with 221 cells provided evidence, that both $CD56^+$ T and NK cells are similarly induced to proliferate upon ligand-binding of CD94/NKG2 receptors during long-term co-culture. To assess if encounter of HCMV infected cells can alter the prevalence of PBL subpopulations in a similar way, PBMC were co-cultured with CMV infected fibroblasts for up to 14 days in the presence of 10 international units recombinant human IL-2, supplemented from day 3 on.
Similar to what was found for 221 co-culture, the response of $CD56^+$ PBL subpopulations was to a large degree dependent on the blood donor. In the limited number of samples studied, HCMV seroprevalence was not indicative for a certain response and further investigation would be necessary to clarify whether latent HCMV infection of a donor would hamper or support amplification of certain cell subpopulations *in vitro*.
For one out of six PBMC donors, strong, HCMV induced proliferation of natural killer cells was found (Fig. 3.16). The amplified NK cells were $CD56^{bright}$, a subset known to be increased

at sites of inflammation (Strowig, et al., 2008). The vast majority (93.5%) expressed NKG2A and a high percentage co-expressed NKG2C (15%, data not shown). Expression of CD16 indicates the potential to perform ADCC and common HLA-DR expression is suggestive of an activated phenotype (Lima, et al., 2001). Taken together, cell surface protein expression of NK cells specifically amplified upon co-culture with HCMV infected cells adumbrates a potential to counteract the virus. In another two PBMC donors, elevated frequencies of $CD56^+$ T cells were found (Fig. 3.17) if MRC-5 cells were infected with an MOI not higher than 1 as PBMC did not withstand co-culture with cells carrying a higher viral load. The appearance of these $CD56^+$ T cells was not accompanied by strong total cell expansion. This could be due to: First, CD56 is found on a subpopulation of T cells which frequently co-express other "NK cell receptors" (NCR) like CD94/NKG2 and was previously even used as a marker for natural-killer T (NKT) cells. NCR^+ T cells were implicated as major contributors to determine the outcome of viral infections (Golden-Mason, et al., 2007). It could be that expansion of this very small initial population could take place. Second, activation-induced upregulation of CD56 could have occurred, which has been shown for example upon CD3/TCR stimulation resulting in increased numbers of $CD56^+$ T cells whilst total cell numbers remain unchanged (Kelly-Rogers, et al., 2006). Third, CD56 expression on T cells might be indicative of senescence of the cells. For example CD56 expression has been implicated with longevity, senescence and exertion of TCR independent immune cascades previously (Lemster, et al., 2008) as well as generally with cytolytic effector functions of $CD8^+$ CTL (Pittet, et al., 2000).

Taken together, the observed increased expression of NKG2C, correlating with the phenotype of highly differentiated cytotoxic T lymphocytes (Arlettaz, et al., 2004), could be indicative of a preferential "survival" of potent $CD3^+$ $CD56^+$ $NKG2C^+$ effector cells which might be preferentially induced in response to HCMV. It has to be mentioned that although fibroblasts are highly sensitive to CMV infection, the relevance of PBMC interaction with fibroblasts for CMV immunosurveillance *in vivo* is not clear. It may be more likely that cells of the vascular system, which had been implicated as potential sites of HCMV latency, are involved and for that reason, this work also provided the basis to test human vascular endothelial cells (HUVEC) as HCMV carriers in an additional co-culture system. To achieve this, a HCMV lab strain more closely related to primary isolates, named TB40, has to be used for infection of HUVEC and corresponding virus stocks were prepared. This will allow to analyze the response of PBMC to HCMV in a biologically more relevant in vitro system, superior to reflect the in vivo situation.

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6. Curriculum Vitae

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