T and NK cell immunity after hematopoietic stem cell transplantation

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Chapter 9

General discussion and future perspectives

In this chapter, the findings as described in this thesis and summarized in the previous chapter will be discussed in the context of the current literature and clinical perspective. First, the interaction of T cell reconstitution and viral infections after HSCT will be discussed. Then, pharmacological and adoptive immunotherapeutic interventions for the protection of viral infections in patients with a delayed T cell reconstitution will be addressed. Thirdly, the role of NK cells in patients lacking T cells will be reviewed. The fourth section of this chapter will focus on the novel lymphoid tissue NK cell population. Then, the possible relationship between the different human NK cell populations will be discussed. The concluding paragraph will summarize the main findings and implications of this thesis and provide perspectives for further research.

1 Interaction of viral infections and T cell reconstitution

Many studies have demonstrated the importance of the rapid reconstitution T cell immunity to achieve sustained control of viral reactivations after HSCT.[37-39] Eventually, T cell reconstitution occurs after every successful HSCT, but little is known about the influence of early viral infections on long term T cell reconstitution, which is addressed in Chapter 2.

The morbidity and mortality associated with herpesvirus infections after HSCT have dropped significantly in the last two decades as a result of the improved pre-emptive therapy. Still, the immunological impact of CMV infections remains an important subject of investigation. This attention is mainly driven by the relation between CMV reactivations and the reduced risk of acute myeloid leukemia (AML) relapse after HSCT.[178;280] Different mechanisms mediating this effect have been proposed. The first hypotheses are based on T cell responses: CMV-antigen presentation on CMV-infected AML blasts or cross reactivity between CMV antigens and tumor antigens both leading to leukemia-killing by (the abundantly present) CMV-specific $CD8^+$ T cells.[178;281] Alternatively, the anti-AML response can be mediated by NK cells as CMV infection leads to an increased expression of ligands for activating NK cell receptors on AML blasts, shifting the balance towards NK cell activation.[129;282]

The second reason for the persistent interest in CMV is the so-called "memory inflation", initially observed in CMV seropositive elderly individuals.[147;148] Furthermore, CMV reactivations in solid organ transplant recipients receiving life-long immunosuppressive medication have been correlated to an accelerated and ongoing accumulation of late differentiated T-cells with a stable contribution of CMV-specific T cells.[154;177] This expansion of CMV-specific T cells is proposed to be induced by continuous or repetitive stimulation by (subclinical) CMV reactivations.[283] Memory inflation is associated with faster immunosenescence, increased systemic inflammation and a higher risk of cardiovascular disease.[283]

Investigation of the reconstitution of an immune system with an appropriate function and a broad repertoire after pediatric HSCT receives more attention nowadays as a consequence of the improved long term survival. After HSCT, $CD8^+$ T cell numbers continued to rise in the months following the clearance of CMV viremia. In the second year after HSCT, a regression of memory T cell numbers was observed (Chapter 2). These dynamics are comparable to the recently published observations of the impact of primary CMV infections in healthy children, as studied in the large Generation R study in Rotterdam.[284;285] Still, the magnitude of the T cell expansion was almost tenfold higher in HSCT recipients compared to healthy children (Chapter 2 and ref[285]). High viral loads are reached during CMV reactivation in the absence of T cell surveillance, resulting in latent infection of large numbers of cells. This strong antigenic stimulation in combination with the pro-inflammatory environment after HSCT could explain the difference between healthy children and HSCT recipients.[143;164;165;286]

In contrast to solid organ transplant recipients or elderly people, no memory inflation was observed after HSCT. Although the amplitude of the early T cell response was high after HSCT, the dynamics of this immune response mimics the normal immune response in healthy individuals, with a plateau phase followed by regression of $CD8^+$ T cell numbers. Also, CMV infection did not have a negative impact on thymic output, as the reconstitution of $CD4^+$ as well

as $CD8^+$ naïve T cells was not affected (Chapter 2). However, longer follow up is needed to evaluate whether disseminated CMV reactivations after HSCT can lead to premature memory inflation and related (immune) pathology in the decades after pediatric HSCT.

2 Bridging the time to T cell reconstitution

The chapters about viral complications and early immune reconstitution (Chapters 3, 4 and 6) focused on the time window between HSCT and the moment of T cell reconstitution. This period is of great clinical interest because patients are at risk for disseminating viral infections during this phase.[37-39] Because T cells are pivotal for the control of viral infections, most immunological and pharmacological interventions are aimed at accelerating T cell reconstitution or otherwise at bridging the time to T cell reconstitution.

2.1 Pharmacological intervention

For different reasons, HSCT recipients with HAdV infections are in a precarious situation. First, HAdV infections after HSCT form a typical pediatric problem, receiving relatively little attention from the general HSCT field. As a result, they do not benefit from research advances made in the adult HSCT setting. Also, in the pediatric population, antiviral drugs are prescribed off-label and large clinical trials needed for the development of novel antiviral agents are difficult to perform. In contrast to CMV and EBV, for which the use of pre-emptive therapy has greatly improved the outcome, the currently available antiviral drug cidofovir does not lead to adenoviral load reduction in the absence of lymphocyte reconstitution. At best, cidofovir resulted in temporal viral load stabilization (Chapter 4). Although this viral load stabilization can bridge the period until T cell reconstitution, in 17% of cases, HAdV viremia progressed despite cidofovir treatment. Additionally, cidofovir is highly nephrotoxic leading to both acute and chronic kidney injury after HSCT, limiting its use (G. Lugthart unpublished data and references [53-56]). In the next few years, the position of HSCT recipients with HAdV infections may improve as a result of the introduction of the novel drug brincidofovir. Brincidofovir is the orally bioavailable lipid conjugate of cidofovir and the first published data are promising, showing an increased effectiveness and a reduced toxicity profile.[60;61] The majority of HSCT recipients receiving brincidofovir as a treatment for HAdV infections had a rapid reduction of HAdV load during brincidofovir treatment.[60;61] Both studies did not report data on lymphocyte reconstitution during treatment, which is a major confounder in studies of drug efficacy. In the study by Hiwarkar *et al.*, brincidofovir was given as primary treatment, and reduction of HAdV load was observed independent of the time after HSCT, suggesting an effect which does not depend on the presence of lymphocytes.[60] Nevertheless, studies correlating HAdV loads with brincidofovir treatment in the absence of lymphocyte reconstitution are needed to confirm these promising data.

2.2 Adoptive transfer of virus-specific T cells

The administration of donor derived virus-specific T cells has been applied with promising results in the treatment of CMV and EBV reactivations after HSCT (reviewed in ref [287]). For HAdV,

success rates of virus-specific T cell therapy are more variable.[288-290] The generation of HAdV specific T cell products is complicated by the low frequency of HAdV-specific T cells in healthy donors, resulting in small doses of transferred virus specific T cells.[288] This was observed in Chapter 3 as well: despite enrichment, the frequency of HAdV specific T cells was low in the multi-virus specific T cell product.

A major limitation of virus-specific T cell therapy is the high level of viral dissemination and presence of HAdV-associated disease at the time of T cell infusion in the therapeutic setting. For this reason, infusion of T cells before progression to disease might be more successful. In Chapter 3, we developed a method to generate a product containing T cells specific for CMV, EBV and HAdV for prophylactic treatment. Whether the standard prophylactic administration of multivirus-specific T cells will become standard of care can be debated. Currently, the evidence for the pre-emptive use of virus-specific T cells is based on phase I studies. Three phase II randomized clinical trials (RCTs) with prophylactic administration of CMV-specific T cells after HSCT have been performed. All three studies finished three years ago. Results of one study were published and results of the second study were presented at the 2014 Annual Meeting of the American Society of Hematology, but have not been published yet. Results were comparable: although patients receiving prophylactic CMV-specific T cells had a lower severity of CMV reactivations and required less antiviral drugs, the incidence of CMV reactivations was not significantly reduced in the treatment arm.[291;292] Results of the third study have not been reported yet. At the moment, a multicenter RCT using multivirus-specific T cells is in preparation (TRACE, initiated by T. Feuchtinger, Tübingen, Germany).

The improvement of virus-specific T cell immunity is particularly needed in patients with an expected delay in T cell reconstitution. Importantly, delayed T cell reconstitution is strongly correlated with the exposure to serotherapy (Chapter 6 and refs [30;33]). If T cell depleting antibodies circulate in the HSCT recipient at the time of administration of the graft, these antibodies will also deplete the donor T cells infused with this graft. This is illustrated by the reduced success rate of HAdV-specific T cell therapy in patients who received serotherapy compared to patients without serotherapy.[288] Therefore, the concentration of circulating ATG or alemtuzumab should be monitored prior to infusion of the T cell product. By the time these antibodies are below the lympholytic level, T cell reconstitution will be eminent, reducing the need for adoptive T cell transfer. In the end, the personalized dosing of serotherapy will prevent overdosing of serotherapy, improve T cell reconstitution and reduce the need for virus-specific T cell therapy.

The number of patients that qualify to receive *prophylactic* multivirus-specific T cells to prevent one serious virus-infection after HSCT may turn out to be high (high number needed to treat). Still, a subgroup of HSCT recipients would in fact benefit from *targeted* adoptive T cell therapy. Identification of these patients will improve the effectiveness of adoptive immunotherapy. In Chapter 4 and 6, we identified subgroups of patients with a poor prognosis which lacked protective T- and NK cell reconstitution. This can be due to high concentrations of serotherapy or high dose steroid treatment because of the occurrence of acute GvHD. However, these patients will not benefit from adoptive immunotherapy as transferred T cells will be susceptible to immunosuppression by steroids or elimination by serotherapy. Other patients with a poor prognosis had graft failure or unexplained slow immune reconstitution. These patients often

require a retransplantation or stem cell boost, but it takes months before protective T cells recover from the newly infused stem cells. For these patients, adoptive transfer of virus-specific T cells should be applied in parallel with a stem cell boost to prevent further dissemination of viral infections.

3 Role for NK cells after HSCT

In healthy individuals, NK cell function may be overshadowed by the presence of T cells. We regarded the treatment induced T cell deficiency after HSCT as a model to study the potential of human NK cells in the absence of T cells.

3.1 Interaction between viral infections and NK cell reconstitution

Various studies highlighted the inferior outcome of patients with a delayed T cell reconstitution after ATG serotherapy as part of the conditioning prior to HSCT.[30;33] In Chapter 6, we demonstrated that leukemia patients with delayed T cell reconstitution after ATG serotherapy together with an expansion of effector $CD56^{bright}$ NK cells did not have an inferior clinical outcome after HSCT compared to patients with rapid T cell reconstitution. In Chapter 4, a reduction of HAdV load was observed in the absence of T cells in patients with an expansion of ($CD56^{bright}$) NK cells. Although it is impossible to discriminate between the relative contribution of cidofovir and NK cells, this suggests that HAdV infection elicits an NK cell expansion, and that an expansion of NK cells can lead to a reduction of the HAdV load. In Chapter 6, we observed a strong correlation between $CD56^{bright}$ NK cell expansion early after HSCT and the occurrence of EBV reactivations. Although this correlation was not statistically significant after multivariate correction for delayed T cell reconstitution, this (preliminary) observation can have immunological significance. As EBV seroprevalence is almost universal, the majority of HSCT recipients will experience a (subclinical) reactivation of EBV in the absence of T cell surveillance. Early after HSCT, NK cell are the only lymphocytes available to respond to viral triggers. Therefore, it is conceivable that EBV and HAdV infections contribute to the expansion of early differentiated NK cells after HSCT. For both viruses, NK cells alone were not able to control the infection. HAdV load reduction was mainly observed in the first weeks of the NK cell expansion. Still, the presence of high numbers of NK cells did not prevent from subsequent (secondary) HAdV dissemination (Chapter 4). Also, the peak of the viral load during EBV reactivations was observed in the weeks after the expansion of NK cells (G. Lugthart, unpublished data). In murine studies, depletion of NK cells was only associated with increased viral loads in the early phase of infection.[116] Our data support the hypothesis that NK cells play a role in the early immune response to viral infections, but are not able to clear these infections on their own. However, a direct relation between (control of) infection and NK cell expansion is hard to prove in HSCT recipients with many different infectious and inflammatory triggers.

In Chapter 6, we did not observe a correlation between CMV infections and the expansion of either $CD56^{bright}$ or $CD56^{dim}$ NK cells early after HSCT. The NK cell phenotype associated with

CMV infections, the late differentiated NKG2C^{+} CD56^{dim} NK cells, was not present during the early phase of CMV infection but expanded between 6 and 12 months after HSCT.[129] This expansion of late differentiated NK cells appears to be secondary and is reminiscent of the expansion of late differentiated CD8^{+} T cells after CMV infections post HSCT (Chapter 2).[129] This marks a major difference with HAdV and EBV infections, where NK cells expand at the early phase of the infection. An extensive phenotyping of NK cells expanding during EBV and HAdV infections after HSCT can be used to identify "virus-specific" NK cell characteristics. In recent years, a number of studies have been published proposing that the NK cells responding during (primary) EBV infections have an early differentiated phenotype. An expansion of CD56^{bright} and early differentiated NKG2A^{+}KIR^{-} CD56^{dim} NK cells was observed in healthy individuals with primary EBV infections,[120;121] as well as after *in vitro* stimulation with EBV infected cells.[293;294] The distinct differentiation of NK cells associated with CMV and EBV infections is reminiscent of the differentiation of CMV and EBV specific CD8^{+} T cells. Whereas CMV-specific CD8^{+} memory T cells are characterized by a late differentiated CD27$^{+/-}$CD28^{-} EM and EMRA phenotype, EBV-specific T cells are mainly found in the early differentiated CD27^{+}CD28^{+} EM compartment.[70]

In a humanized NOD SCID gamma (NSG) mouse model, an expansion of NK cells was observed upon EBV infection, and depletion of NK cells resulted in higher viral loads and more severe disease.[295] Unfortunately, the study of human NK cell biology in this humanized NSG mouse model is complicated because of the lack of CD56 expression on NK cells.[295;296] *In vitro* experiments using activated, early differentiated NK cells from HSCT recipients can be used to investigate whether these cells have an increased potential against Epstein-Barr virus-transformed lymphoblastoid cell lines in comparison to healthy donor NK cells. Still, the complexity and combined problems of HSCT recipients make it hard to convincingly demonstrate virus driven NK cell responses. For this, immune monitoring during primary EBV infections in healthy individuals would be more suitable, yet this is hard to realize in view of practical and ethical considerations. In the follow-up of primary EBV infections, the first week of the immune response, during which NK cells might play a major role, will be missed. An alternative approach to increase the understanding of human NK cell immunity to EBV could be the simian lymphocryptovirus infection model in rhesus macaques.[297;298] This virus is very similar to the human EBV, whereas macaque NK cells are more comparable to human NK cells than commonly used murine NK cells.[297;298]

3.2 Compensatory adaptations of NK cells during T cell deficiency

The functional profile of post-transplant CD56^{bright} NK cells corresponds with that of conventional, cytokine secreting CD56^{bright} NK cells that have temporarily acquired cytolytic potential (Chapter 6). Other studies demonstrated a low cytotoxicity of post-transplant CD56^{bright} NK cells, but in these studies the cells were subjected to prolonged *in vitro* culture before performing cytotoxicity assays or purified CD56^{bright} cells acquired at three months after HSCT were investigated.[136;216;299] By that time, the granzyme B expression of CD56^{bright} NK cells was already strongly reduced (Chapter 6).

CD56^{bright} NK cells early after HSCT had an increased expression of receptors associated with migration towards inflamed tissues and skin, but were circulating in blood. The fact that these cells are circulating implies that the expression of chemokine receptors alone is not enough to retain these cells in tissues. We did not investigate whether CD56^{bright} NK cells were further enriched in inflamed tissues or skin, which could improve the understanding of the data presented in Chapter 6. The chemokine receptor expression profile on circulating NK cells might be a reflection of the total circulating and non-circulating NK cell pool. Possibly, these cells are traveling towards tissues, or have just left the tissues. The circulating NK cells early after HSCT did not express CD69. Although CD69 was initially identified as an early lymphocyte activation marker,[264-266] CD69 has been increasingly recognized to play a role in tissue retention. This is mediated via the downregulation of sphingosine-1-phospate receptor 1 (S1PR1), which is needed for cells to return to the blood stream.[300;301] Possibly, the inflammatory environment after HSCT results in the expression of chemokine receptors to increase their potential to rapidly access sites of inflammation. Once the cells enter their target tissue, the local expression of activating cytokines might engage CD69 expression to facilitate the retention in these tissues.

The expression of inflammatory chemokine receptors, combined with a strong IFN-γ production and fully equipped cytotoxic machinery, enables CD56^{bright} NK cells to respond rapidly in the early phase of viral infections after HSCT. Whether these cells expand in response to viruses, or as a result of general inflammation and cytokine storm remains to be elucidated. The strong expansion of CD56^{bright} NK cells with acquired functional assets of CD56^{dim} NK cells occurred in the presence of CD56^{dim} NK cells. Why do cytotoxic CD56^{dim} NK cells not expand themselves? Possibly, CD56^{bright} NK cells are a more sensitive to exogenous stimulation. This corresponds to the high proliferative capacity of *in vitro* activated healthy donor CD56^{bright} NK cells compared to CD56^{dim} cells.[97] Alternatively, *in vivo* activated CD56^{dim} NK cells left the blood stream, or acquired a CD56^{bright} phenotype, as has been reported for *in vitro* activated CD56^{dim} NK cells.[105]

Our observations are in line with the limited reported observations in patients with severe inborn T cell deficiencies. In patients with RAG and ARTEMIS deficiencies, an increased proportion of NK cells had the CD56^{bright} phenotype, and these cells displayed a high degranulation upon coculture with K562.[235] An increased proportion of CD56^{bright} NK cells has been reported in patients with T cell receptor-γ, -δ and -ζ deficiencies as well.[302] In a case report describing an X-linked SCID patient with the rare T-B+NK+ phenotype, an expansion of CD56^{bright} NK cells was described.[303] In this patient, all skin infiltrating lymphocytes were CD56^{bright} NK cells. This would match with the chemokine receptor expression profile observed after HSCT, with a temporarily increased expression of cutaneous lymphocyte antigen (CLA) on CD56^{bright} NK cells. Finally, also in human immunodeficiency virus (HIV) infected patients with an acquired T cell deficiency, an inverse correlation was observed between CD4^{+} T cell numbers and the proportion of NK cells with the CD56^{bright} phenotype. Like post-transplant CD56^{bright} NK cells, these cells had a reduced expression of CCR7 and an increased expression of granzyme B.[236] Together, these data demonstrate the flexibility of the human immune system and the potential of the minor CD56^{bright} NK cell population in situations when T cells are not able to perform their task.

4 Identification of a lymphoid tissue resident NK cell subset

Most knowledge on the phenotype and function of NK cells is derived from cells present in blood. In the blood of healthy donors, $CD56^{bright}$ NK cells form a minor non-cytotoxic NK cell subset. In this thesis, we describe two situations in which $CD56^{bright}$ NK cells form a major cell subset with distinct phenotypic and functional properties. Using 12-color flowcytometry, we demonstrated that $CD56^{bright}$ NK cells expanding early after HSCT are distinct from $CD56^{bright}$ NK cell in steady state conditions (Chapter 6 and discussed in the previous section). Also, we discovered a third, phenotypically and functionally distinct NK cell population in lymphoid tissues (Chapter 7). A relative increase of $CD56^{bright}CD16^{+/-}$ NK cells in lymphoid tissues has been discovered decades ago.[108;216] The use of CD56 and CD16 to discriminate the two main ($CD56^{bright}$ and $CD56^{dim}$) NK cell populations originates from the era of 4-color flowcytometry. Many characteristics of circulating and easily available $CD56^{bright}$ NK cells have been extrapolated to draw conclusions about NK cell biology in tissues. Without the use of additional markers, this lymphoid tissue residing NK cell population (ltNK) could previously not be discriminated from conventional $CD56^{bright}$ and $CD56^{dim}$ NK cells. In future, the use of mass spectrometry (CyTOF®), measuring ~30 extracellular or intracellular proteins per cell, and single cell RNA sequencing, measuring the gene expression of ~10.000 genes will further increase this resolution.[304;305] Combined with advanced computerized data analysis, these new technologies will enable grouping and unveil relations between immune cell subsets without the investigator bias inevitably associated with the manual gating of cells which is currently applied.

4.1 Tissue retention and Mobility

Important mechanisms for tissue homing and retention are the engagement of chemokine receptors and adhesion molecules. ltNK cells have a distinct expression of chemokine receptors and adhesion molecules in comparison to circulating $CD56^{bright}$ NK cells from healthy individuals.[306-308] The discriminative cell surface markers used to identify ltNK cells are CD69 and the chemokine receptor CXCR6 (Chapter 7). Although CD69 was first identified as an activation marker, CD69 is now recognized to play an important role in tissue retention (see above).[300;301] Simultaneously with our discovery of $CXCR6^{+}(CCR5^{+})$ ltNK cells, a $CXCR6^{+}CCR5^{+}$ human liver resident NK cell population was identified.[261;271] The chemokine receptors CXCR6 and CCR5 were also found to be expressed by murine liver resident NK cells and human lymphoid tissue, liver and lung resident T cells.[269;270;273;309-312] These observations support the hypothesis that these chemokine receptors are involved in tissue residency. Specialized tissue resident NK cells were first identified in the uterus.[313-315] Human uterine NK cells do not express CCR5, whereas their expression of CXCR6 has not been assessed. Instead, uterine NK cells uniformly express the adhesion molecule CD49a, while a proportion of uterine NK cells express CD103 as well.[313-315] These adhesion molecules are expressed by tissue resident NK cells in tonsil, T cells in lung and mucosa and mucosal innate lymphoid cells as well.[262;311;316] However, ltNK cells in bone marrow, spleen and lymph node, and the majority of human liver resident NK cells do not express these markers.[261;271] This suggests that mucosal and non-mucosal tissue resident lymphocytes apply a different mechanism of tissue retention. Interestingly,

(lymphoid) tissue-resident CD56^{bright} NK cells lack the expression of CD62L (L-selectin) and CCR7, involved in recruitment of circulating lymphocytes to lymphoid tissues via high endothelial venules (HEVs).[107;271;313] Apparently, these receptors are not required for the retention of ltNK cells in lymphoid tissues. The uniform evaluation of chemokine receptor and adhesion molecule expression on NK cells from a broad range of human tissues could further improve the understanding of NK cell tissue residency and identify tissue specific characteristics.

4.2 Function and Interactions

Although ltNK cells appear to have a hypofunctional profile in resting state, they produce interferon-γ and become cytotoxic after the appropriate *in vitro* stimulation (Chapter 7). In line with their specific tissue localization and phenotype, it is plausible that these cells have a local function that differs from the classical NK cell functions. Alternatively, they may form a reservoir of NK cells waiting for mobilization, which lose their tissue-retention and CD69^{+}CXCR6^{+} phenotype upon activation. In view of their similarities with liver resident NK cells, CD69^{+}CXCR6^{+} NK cells can be present in other tissues with an enrichment of CD56^{bright} NK cells.[106-108] They can have a more general tissue-residing immune scavenger function protecting the local environment from invading pathogens. Uterine NK cells were shown to produce chemokines and angiogenic factors, suggesting a tissue-specific role in embryonic implantation.[314;317;318] Like other innate lymphoid cell types , NK cells in different tissues can have their own tissue specific function.[88] This can be addressed by the in parallel investigation of tissue resident NK cells from different tissues.

Murine liver (but not spleen) resident CXCR6^{+}CD49a^{+} NK cells have been shown to be involved in hapten- and virus specific memory NK cell responses.[272;273] In both mice and man, virus-specific T cells are enriched in the target-tissue of the respective virus (*e.g.* hepatitis virus in liver, EBV in bone marrow, influenza virus in lung).[270;319-322] Whether human tissue-resident NK cells play a role in such memory responses remains to be elucidated. We did not observe a difference between the size of the ltNK cell population in pediatric and adult bone marrow donors, arguing against the accumulation of an (antigen) experienced cell population (G. Lugthart, unpublished observation).

Based on the localization in lymphoid tissues, it is conceivable that ltNK cells play a role in the enhancement and polarization of adaptive immune responses.[108;253] Clues for the interactions of tissue resident NK cells with other cells may further be deduced from the nature of CXCL16, the exclusive ligand for CXCR6.[323;324] In contrast to most other chemokines, CXCL16 is expressed as a cell surface bound molecule which can be shed to function as a chemo-attractant. CXCL16 functions as a receptor for oxidized lipoprotein and bacteria, but has also been demonstrated to be involved in cellular interactions.[325-328] CXCL16 is expressed by both immune cells and other cells. For example, dendritic cells (DC) in T cell zones of the lymph node, liver sinusoids and –still unidentified– cells in the splenic red pulp express CXCL16. In concordance, NK cells have been shown to co-localize with DCs in T cell zones of lymph nodes and are mainly found in liver sinusoids and in red pulp of the spleen.[108;256;257;271] Fluorescence immunohistochemistry and confocal microscopy should be used to determine the exact tissue localization of ltNK cells and their interactions with the surrounding cells for a better understanding of their interactions and

possible function. In addition, gene expression data will unravel signaling pathways and transcription factors involved in tissue residency, as has been shown for tissue-resident T cells.[311;329] Furthermore, gene expression analysis can identify pathways involved in cellular processes to provide clues about their function and interactions.

It has been suggested that CXCR6-CXCL16 interactions play a role in the enhancement of immune-responses[330;331] Recently, it was shown that co-culture with $CXCL16^+$ activated dendritic cells stimulated $CXCR6^+$ murine NKT cells to produce IFN-γ.[332] When this applies for NK cells, ltNK cells might play a role as a catalyst for the generation and polarization of $CD4^+$ Th1 immune responses and $CD8^+$ T cell responses in lymphoid organs. Based on the cellular interactions and gene expression data, targeted functional experiments can be designed to improve the understanding of the role of ltNK cells in human immune responses.

5 Relationship between different NK cell subsets

The skewing towards $CD56^{bright}$ NK cells after HSCT and the expression of the early differentiation markers CD27, CD117 and CD127 on $CD56^{bright}$ NK cells, but not $CD56^{dim}$ NK cells from healthy donor blood have led to the hypothesis that $CD56^{bright}$ NK cells are the precursors of $CD56^{dim}$ NK cells.[91;216;299] However, in Chapter 6, we show that the reconstitution of $CD56^{dim}$ NK cells after HSCT occurred independently of $CD56^{bright}$ NK cell- and T cell reconstitution. Furthermore, $CD56^{bright}$ NK cells early after HSCT did not have an immature, but rather an activated phenotype. This corresponds to the high proliferative capacity of *in vitro* activated healthy donor $CD56^{bright}$ NK cells compared to $CD56^{dim}$ cells.[97] Various studies reported the existence of phenotypic and functional intermediate stages in the progression from $CD56^{bright}$ to $CD56^{dim}$ NK cells in peripheral blood of healthy donors and patients after HSCT based on the expression of CD16, CD27 and CD117.[100;126-128;279;333] Notably, the increased expression of CD16 and reduced expression of CD117 and CD27 on $CD56^{bright}$ NK cells can be affected by cytokine-activation.[100;126;127;279;334] Therefore, the dynamics and phenotype of NK cell reconstitution in the inflammatory conditions early after HSCT might be more informative about the response of different NK cell subsets to *in vivo* stimulation rather than about their developmental (inter)relation.

The distinctive reconstitution profile of ltNK cells after HSCT (Chapter 7) indicates that these cells form a distinct subset with a limited expansion upon exogenous stimulation. Their recovery followed the reconstitution of $CD56^{bright}$ and $CD56^{dim}$ NK cells, arguing against a position as NK cell precursor-population or an intermediate between $CD56^{bright}$ and $CD56^{dim}$ NK cells. Another argument to reject the hypothesis that ltNK cells could be a precursor population, is the lack of expression of the early differentiation markers CD117 and CD127 on ltNK cells. It is unlikely that the lack of expression of these markers is caused by cellular activation, because the tissues in which ltNK cells were identified were harvested from a non-inflammatory environment in healthy donors.

The discovery of distinct tissue-resident CD56^{bright} NK cell populations increases the number of possible relationships between the NK cell populations. Data from transcription factor-deficient mice suggested that circulating and tissue-resident NK cells are derived from different cell lineages.[335] However, the lack of CD56 expression on murine NK cells hampers the direct comparison to human NK cell populations. Transcriptome analysis comparing human uterine NK cells with both circulating CD56^{bright} and CD56^{dim} NK cells highlighted major differences in gene expression profile between the three NK cell populations.[313] Gene tracking data in rhesus macaques transplanted with lentiviral barcoded hematopoietic stem cells demonstrated that the lineage origin of the macaque NK cell homologues of CD56^{bright} (CD56^{+}CD16^{-}) and CD56^{dim} (CD56^{-}CD16^{+}) NK cells is different, suggesting that CD56^{dim} NK cells do not develop from the CD56^{bright} population.[298]

Rare patients with human NK cell deficiencies may provide clues about the developmental relationship between CD56^{bright} and CD56^{dim} NK cells as well. No mutations in transcription factors have been described as a putative cause of the lack of CD56^{dim} NK cells while the CD56^{bright} NK cells are spared. Patients with a partial minichromosome maintenance complex 4 (MCM4) deficiency, a molecule involved in proliferation, have reduced numbers of circulating CD56^{dim} NK cells and normal numbers of CD56^{bright} NK cells.[336] Mutations in the transcription factor gene *GATA2* result in the absence of CD56^{bright} NK cells, while CD56^{dim} NK cells are still present.[337] Together, these observations argue against the hypothesis that CD56^{dim} NK cells are derived from CD56^{bright} NK cells. The distinct phenotype and functional signature of ltNK cells supports the hypothesis that these NK cells develop locally, independently of the circulating NK cells. Nevertheless, additional studies are needed to shed new light on the developmental relationship between CD56^{bright}, CD56^{dim} and tissue-resident CD56^{bright} NK cell populations and their precursors. Gene-expression data can be used to identify transcription factors that are preferentially expressed in one of the NK cell populations. The evaluation of ltNK cells in the bone marrow aspirations from patients with known defects of genes that play a role in the immune system could identify the impact of these genes on resident NK cells. Finally, life-span and relation of circulating and tissue resident NK cell subsets could be further studied by infusion of radioisotope labelled NK cell subsets in (humanized) animal models.

6 General conclusions

In this thesis, new insights in the complex and intertwined relationship between viral infections, T cells and natural killer cells after allogeneic HSCT in children are provided and discussed. Patients are at high risk of viral complication during the T cell deficient period early after HSCT. When viral infections occur, interventions to bridge the period until the recovery of antiviral T cell immunity are of great importance to improve the clinical outcome after HSCT. As a result of ongoing developments in the field, the perspective of some of the chapters may soon change. For example, the use of cidofovir for the treatment of HAdV infections may soon be history because of the introduction of the new (and promised to be better) drug brincidofovir. However, also brincidofovir should also be critically evaluated and related to HAdV load dynamics in the absence of lymphocyte recovery. The prophylactic use of adoptive T cell therapy for multiple viruses might never become the standard of care for pediatric HSCT recipients because of the improved understanding of the *in vivo* effectiveness and pharmacodynamics of serotherapy agents. These developments will result in personalized dosage schedules, reduction of over-dosage and a reduction of patients with a prolonged T cell deficiency after HSCT. Patients who lack T cell recovery in the absence of circulating serotherapy agents might benefit from targeted use of adoptive T cell therapy and should be identified. In addition to the importance of T cell reconstitution for the protection of viral reactivations, early CMV infections can explain the often observed severely skewed $CD8^+$ T cell memory compartment in the years after HSCT. However, we also showed that this is caused by an expansion of late differentiated T cells on top of an otherwise balanced immune system. Still, CMV reactivations after HSCT should be included in the late-effect evaluations of pediatric HSCT recipients reaching adulthood, to study its effect on (immune) pathology that might occur decades after HSCT.

Besides antiviral medication and the use of adoptive immunotherapy, NK cells may play a role in the protection against viruses when T cells are not available to perform their task. A strong expansion of $CD56^{bright}$ NK cells occurred in patients with a delayed T cell reconstitution, and these cells displayed functional and phenotypic adaptations. The correlation of specific viral infections with the expansion and function of distinct NK cell phenotypes after HSCT should be explored further, as the period of T cell deficiency after HSCT can be used as a model to improve the understanding of the role of NK cells in antiviral immunity. The identification of CD69 and CXCR6 to distinguish the distinct lymphoid tissue resident ltNK cell population from circulating cells enables the further characterization of the interactions of tissue-resident and circulating NK cells with other immune cells in lymphoid tissues. For example, gene expression analysis comparing tissue-resident and circulating NK cells can provide clues about the specific functions of tissue-resident NK cells, while immunohistochemistry and confocal microscopy may reveal the cellular interactions of tissue-resident NK cells. Importantly, both ltNK cells in lymphoid tissues and $CD56^{bright}$ NK cells early after HSCT have a distinct phenotype and function compared to conventional ($CD56^{bright}$) NK cells. These data illustrate that human NK cell biology goes beyond the current models which are mainly based on circulating NK cells from healthy individuals. This invites for a re-assessment of the relationship between the different human NK cell populations and their role in human immunity.