Immune response to herpesvirus infections in immunocomprised children
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Chapter 9

Summary & General Discussion
SUMMARY AND GENERAL DISCUSSION

The vast majority of clinical problems with primary and reactivated herpesvirus infections arise in immunocompromised patients who cannot adequately contain herpesviruses by themselves, resulting in a high incidence of morbidity and mortality. Primary or acquired defects of the immune system may lead to inadequate immunity to herpesviruses. In this thesis, we have focused on children receiving immunosuppressive medication, either after solid organ transplantation, or in the course of anti-cancer treatment. The scope of this thesis is to reveal factors that contribute to impaired immunity to herpesviruses in immunocompromised children. To understand the specific deficits in immune responsiveness to herpesviruses in these patients, we need to know how the immune system deals with these viruses under physiological circumstances both in the acute phase of infection and during latency. These insights will help to predict which patients are at risk to develop severe herpesvirus-induced disease as well as to provide preventive and therapeutic patient-tailored strategies.

Containment of herpesviruses is critically dependent on the concerted action of NK cells, B cells and virus-specific CD4+ and CD8+ T cells [1-4]. T cells can be divided into functional subsets by specific surface markers, such as CD27, CCR7, CD45RA, and CD45R0 [5-8]. CD45RA+CD27-CD8+ T cells were considered to be terminally differentiated T cells [9,10]. In HIV infection, HIV-specific T cells bearing this phenotype could not be detected and the conclusion was drawn that skewed maturation of CD8+ T cells would account for the incapability of infected patients to control the virus [9,11]. The study described in Chapter 2 shows that a highly significant correlation was found between the percentage as well as the absolute number of circulating CD8+CD45RA-CD27- cytolytic T cells and CMV seropositivity in healthy individuals. This was not observed for EBV or VZV (and measles, mumps, or rubella). The number of CD8+CD45RA+CD27- T cells remained surprisingly constant when studied longitudinally for 2-4 years. The expansion of CD8+CD45RA-CD27- T cells in peripheral blood was clearly triggered by CMV infection, since the numbers of these cells increased during acute CMV infection in children without known immune disorders, starting within the first month after diagnosis. Within 2 months a plateau was reached with stable numbers of CD8+CD45RA-CD27- T cells for the following years. In agreement with previous findings [12,13], CMV-specific cells were never detected in the naive (i.e. CD45RA-CD27bright) subset of CD8+ T cells. Enrichment of the CMV peptide-specific T cells was particularly observed in the CD27- pool of CD8+ T cells, as well as in the CD27dul fraction [14,15]. The mechanism whereby CMV induces a large number of uniquely expanded CD8+ cytolytic T cells warrants further study as an intriguing facet of the interplay between CMV and the immune system. Interactions between CD27 and CD70 may provide a clue. CD27 is down-regulated on interaction with its cellular ligand, CD70. Induction of CD70 may vary in distinct viral infections explaining the different phenotypes of virus-specific CD8+ T cells with regard to CD27 expression [16]. Further to this we recently found that CMV not only influences the composition of CD8+ T cell subsets, but also of CD4+ T cell subsets, since a high frequency of CD4+CD28+ T cells can exclusively be detected in CMV-seropositive individuals [17].
Resolution of primary EBV infection and protection from reactivation is to a large extent dependent on control by virus-specific CD8⁺ T cells. Because of immunosuppressive medication which is widely used in transplant patients, primary infection and reactivation of EBV are associated with development of posttransplant lymphoproliferative disease (PTLD) [18,19]. The interplay between EBV and host immune factors can be evaluated by assessing "spontaneous" EBV transformation of B cells [20]. In Chapter 3, we designed a prospective study in a cohort of pediatric renal transplant patients on different regimens of immunosuppressive drugs to evaluate the use of the SET assay (i.e. "spontaneous" EBV B cell transformation-assay) as a measurement of the potential infectious activity of EBV in face of the anti-viral activity of the T cell system.

Most importantly, EBV-specific CD8⁺ T cells were detectable in blood from patients with negative SET assays (i.e. no proliferation of cells), coinciding with significantly lower EBV loads, whereas these cells were less frequent in blood from patients with positive SET assays. The relevance of the SET assay was demonstrated by the fact that immunosuppression seemed to be directly linked to B-LCL generation in vitro, as illustrated by a patient in whom reduction of medication resulted in a complete normalization of the subsequent SET assays. We therefore believe that the SET assay is a reflection of the interplay between viral replication, transformation of B cells, and EBV-specific immunity in vivo and hence a valuable screening test for EBV-driven lymphoproliferative phenomena in allograft recipients. Current PCR assays to quantify viral load do not discriminate between infectious virus and DNA fragments. Therefore, determination of viral DNA to predict the risk of development of PTLD is probably only indicative in combination with data on EBV-specific T-cell immunity [21]. The SET assay enables physicians to reduce immunosuppressive medication in a patient-tailored approach.

Not only EBV infection, but herpesvirus infections in general are a threat to immunocompromised patients. Vaccination of patients at-risk, such as children with a malignancy, may protect these children from development of serious disease. Currently, varicella vaccine is the only approved vaccine to prevent illness from herpesvirus infections. The incidence of morbidity and mortality from primary varicella-zoster virus (VZV) infection is increased in immunocompromised children. Vaccination of VZV-seronegative cancer patients with live attenuated varicella vaccine has proven to be safe and effective [22-26]. However, in these studies the vaccine was administered either before the start of chemotherapy, or during maintenance therapy. Postponing chemotherapy can have its impact on clinical recovery, whereas vaccination during maintenance therapy may simply be too late. In Chapter 4 we describe the efficacy of a single dose of VZV vaccine administered to eleven seronegative pediatric oncology patients without interrupting chemotherapy and introducing the vaccine in an early phase (within three months after the start of chemotherapy). Seroconversion was detected in 8 of the patients (72.7%) after vaccination. Seroconversion did not correlate to the age at vaccination, the time between start of chemotherapy and vaccination, nor with the number of circulating lymphocytes at the time of vaccination. Mild adverse effects were observed in five of the patients (45.4%). According to the clinical score of Vazquez et al. [27], all patients scored maximal 2 points and were considered to have mild disease. The efficacy of vaccination was demonstrated upon household contact to wild-type VZV, as documented for two of the patients. Varicella vaccination protected one of the patients completely from development of varicella. Interestingly, seroconversion could not be observed in this patient after vaccination, neither after exposure to the wild-type virus.
other patient was protected from developing severe disease, as historically observed in unvaccinated patients.

In three of the patients, VZV DNA loads were detectable after vaccination in peripheral blood and in throat swabs of two of the patients within 6 weeks after vaccination. No household exposures to the wild-type virus were documented for these children during this period. The nasopharyngeal cultures remained negative, which demonstrates that the vaccinated patients did not shed any infectious virus and were therefore not contagious. Virus-specific CD4+ T cells could only be detected after exposure to the wild-type virus, but not in case of seroconversion or vaccination-associated rash. Altogether, the data suggest a suboptimal induction of adaptive immunity upon immunization, rather than primary vaccine failure. Since it has not been known so far whether VZV-specific CD4+ T cells are induced at all on vaccination (for example in healthy children), firm conclusions cannot be drawn at this point. Another interesting aspect of VZV vaccination is the question whether the virus develops latency and may reactivate to cause herpes zoster. If the vaccine-strain is able to establish latency, herpes zoster cases may be more common in vaccinated immunocompromised patients, than in vaccinated healthy children. However, probably only those children that developed a rash upon vaccination, enabling retrograde axonal transport of the virus to the dorsal root ganglia, are at risk of developing herpes zoster [26]. Long-term follow-up of our patients will provide more insight in this matter. Moreover, larger cohorts of pediatric oncology patients will be required to determine the benefit of our vaccination strategy compared with the strategies studied so far.

The ultimate goal of vaccination is the induction of immunological memory, a hallmark of adaptive immunity. On re-infection, a faster and adapted immune response ensures quick control of the virus, often without development of the typical symptoms associated with primary infection. Maintenance of immunological memory is the basis for the existence of typical "childhood diseases". The study described in Chapter 5 provides a detailed analysis of a human virus-specific memory response. Boosting of VZV-specific CD4+ T cells was observed after re-exposure of immune adults upon household contact with children experiencing chickenpox. The detection of high levels of these cells probably results from a combination of proliferation and redistribution from regional lymph nodes at the site of infection to the blood. Phenotypic characteristics of the VZV-specific cells, the majority being CD45RA-CD27+, resembled those of EBV- and HCV-specific CD4+ memory T cells [28,29]. Effective memory responses to VZV were maintained for decades, since the grandparent included in this study was protected from developing disease on re-exposure to VZV by her grandchild. Regular boosting by specific antigen may be of prime importance to maintain the memory pool. Boosting of memory responses to VZV may not only be valuable upon re-exposure to the virus, but also to control virus reactivating from latency. The decline in VZV-specific T cell-mediated immunity that occurs with increasing age strongly correlates to the striking increase in frequency and severity of herpes zoster in the elderly [30-34]. The importance of boosting to protect the individual from reactivation, has been suggested by an epidemiological study of Thomas et al. [35], which is in accordance with a hypothesis postulated by Hope-Simpson in 1965 [36], stating that cases of herpes zoster were less frequent in individuals with documented exposures to VZV for the last decade. In most instances, the immune response induced on primary VZV infection is capable of containing the virus. The classical symptoms of varicella, such as a vesicular rash and fever disappear within 1 week after the onset of the disease. Although it has been recognized that
primary VZV infection may result in severe courses of varicella in the immunocompromised, little is known on the development of severe courses of varicella in otherwise healthy children. The vast majority of otherwise healthy children experience an uncomplicated course of varicella. However, we describe in Chapter 6 five otherwise healthy children with a life-threatening course of varicella. After more than 4-12 weeks of intensive treatment because of their clinical condition, four of the children recovered, whereas the fifth child died of multi-organ failure.

Most strikingly, NK cells and primed CD8+ T cells, two key players in immune responses to herpesvirus infections [1,37], were nearly absent from the circulation during the early phase of primary VZV infection. For several reasons such as detection of granzymes, proliferation on IL-15 stimulation, and appearance of the cells during the late phase, we concluded that the absence of these cell populations was not the result of a severe primary defect in the generation or maturation of these cells. We believe that the absence of these cytotoxic cells from the circulation may be the consequence of their redistribution to target sites. The expression of chemokine receptors on the majority of NK cells and primed T cells (e.g. CCR5+CXCR3+CCR7+) enables them to migrate from the circulation to inflamed tissue to eliminate virus-infected cells [7,38-43]. This migration may lead to exclusion of these cells from the circulation, as suggested by several other studies in mouse models, and on hepatitis B and C virus infections in humans [14,44-48]. Secretion of high levels of chemokines at the inflamed sites due to an overwhelming immune response to VZV in these patients, may have led to migration of cytotoxic cells from the blood towards these sites. In addition, the ongoing infection may have disturbed the balance between generation of these cells and apoptosis leading to the exhaustion of the cells [49-51].

The absence of primed CD8+ T cells and NK cells from the circulation during life-threatening varicella as observed in our patients seems at present a unique property of VZV, since in acute CMV- and HIV-infection primed CD8+ T cells are detectable in the circulation [52,53]. In similarity to observations in CMV and HIV infection, the appearance of virus-specific CD4+ T cells was delayed compared with patients experiencing mild varicella and appeared in the circulation upon control of the virus by antiviral therapy [52,54].

Another example of an unusual course of varicella infection observed in an otherwise healthy child is described in Chapter 7. In immunocompetent children, peak viral loads during primary VZV infection can be detected in peripheral blood near the onset of the typical vesicular rash. VZV DNA concentrations normally diminish over time and become undetectable within three weeks after the appearance of the exanthem. The boy described in this study was admitted with severe varicella, consisting of a generalized vesicular rash, high fever, signs of dehydration, and clinical symptoms compatible with pneumonitis. Abnormally high viral loads (>340,000 copies/ml) were found in his blood [55,56]. The DNA concentrations in our patient remained high for at least 1.5 years, whereas regularly VZV DNA is detectable during the first two weeks after the onset of varicella, where after the virus is cleared from blood while developing latency in neurons of the dorsal root ganglia [56-58]. During follow-up, the patient suffered from recurrent (sub-) febrile episodes and non-healing skin ulcers for months after discharge. Clinical recovery preceded complete clearance of the virus. NK cells and CD8+ T cells were activated during the acute infection, and VZV-specific CD4+ T cells were detected at high frequencies. Cellular fractionation of PBMCs over time indicated that VZV DNA was initially detected in B cells, NK cells, and both CD4+ and CD8+ T
cells, as also described by Ito et al. [59]. By contrast, the virus was primarily located in CD8+ T cells during the chronic phase of VZV DNA detection. To our knowledge, chronic VZV following a severe primary VZV infection in an immunocompetent child, based on the abnormal persistence of viral loads in peripheral blood is a new entity of VZV infection. In rare instances when viral loads are high, the virus may develop an aberrant state of latency in immune cells, in which the localization of the virus in different cell types during follow-up may be a reflection of their half-lives. If latency-specific genes would exist, micro-array analysis could reveal whether the virus developed a state of latency in the immune cells of our patient. Unfortunately, these genes have not been identified and described so far.

At this point, we cannot provide a clear-cut answer as to why some children develop such a severe and unusual course of varicella. The course of the disease may be dependent on the combination of multiple factors, such as age, infectious dose, duration of antigen exposure, and genetic factors. The genes encoding a large part of the NK cell receptors are clustered in the so-called natural killer gene complex (NKC). Scalzo et al. found that the NKC-linked autosomal dominant genetic locus known as Cmv1 is responsible for the genetic resistance of certain mouse strains to MCMV infections [60,61]. Cmv1 was shown to encode the NK cell receptor Ly49h in the C57BL/6 mice [62-64]. Therefore, functional polymorphisms in the NKC genes may account for differences in susceptibility to infection in humans.

We know that NK cells play a pivotal role in the immune responses to herpesviruses [1,37,65]. However, knowledge on the generation, maturation and precise activation or function of these cells is still quite primitive in comparison with what has been known for example for T cells. New insights may be helpful in clinical diagnostics of the innate immune response. Recently, two functional human NK cell subsets based on the differential expression of CD56 have been described [66,67]. Since CD27 has been shown to discriminate functional T cell subsets [38], we investigated in Chapter 8 whether this TNF-receptor could also be used to identify functional NK cell subsets. In contrast to most T cells, only a minority of circulating NK cells expressed CD27. NK cell subsets defined by CD27 partially overlap with subpopulations classified on basis of CD56 expression and the expression of NK cell receptors as well as the expression of cytolytic proteins revealed that CD27- and CD27+ NK cells are separate entities.

The distribution of NK cell subsets in peripheral blood may differ from that in lymphoid organs, as observed for T cell subsets [14,44,46]. In line with the partial overlap between CD56- and CD27-defined subsets, significantly higher frequencies of CD27+ NK cells were found in secondary lymphoid organs (53% ± 14%), and in tonsils (38% ± 7%) and the majority of these cells were CD56bright. Furthermore, the local cytokine environment may influence the composition of NK cells. Stimulation of peripheral blood NK cells with IL-15 in vitro resulted in NK cells which largely resembled circulating CD27- NK cells in vivo, whereas stimulation with IL-21 in vitro resulted in NK cells with resemblance to circulating CD27+ NK cells. The balance between these cytokines may thus substantially contribute to the phenotype of NK cells.

Different scenarios on the development of CD27- and CD27+ NK cells can be envisaged, as depicted in Figure 1. We know that the expression of CD27 is differentially regulated on lymphocytes. Early B cells are CD27-, whereas early T cells are CD27+. B cells start to express this TNF-receptor during differentiation, whereas T cells loose the expression of this
receptor. In analogy with B cells, NK cell subsets can derive from a common CD27⁻ NK cell precursor (Fig. 1A). The expression of homing receptors such as CCR7 on these early NK cells may direct the cells to different compartments, such as lymph nodes (CCR7⁺ NK cells) or tissue (CCR7⁻ NK cells), where the local cytokine environment influences the expression of CD27 ("Cytokine-induced differentiation"). In analogy with T cells, all NK cells may develop from a CD27⁺ NK cell precursor (Fig. 1B). Interaction with activation-induced CD70 can downmodulate CD27 expression [68,69] ("Ligand-induced differentiation"). This scenario is less plausible, since the majority of NK cells within umbilical cord blood lack CD27. Alternatively, CD27⁻ and CD27⁺ NK cell subsets may be derived from different precursor cells (Fig. 1C). In such a scenario CD27⁻ NK cells largely remain in the periphery, whereas CD27⁺ NK cells migrate to lymph nodes. Within the lymph nodes, CD27⁺ NK cells may interact with CD70 provided by activated dendritic cells (DC) or B cells [70,71], leading to activation and transient downmodulation of the expression of CD27 [72-74]. These activated CD27⁻ NK cells that are then phenotypically indistinguishable from true CD27⁻ NK cells, may enter the circulation and perform effector functions ("Dichotomous differentiation").

**Figure 1 Models of NK cell subset differentiation.** (A) CD27⁻ and CD27⁺ NK cells may differentiate from a common CD27⁻ NK cell precursor. Expression of homing receptors such as CCR7 on these early NK cells may direct the cells to different compartments, such as lymph nodes (CCR7⁺ NK cells) or tissue (CCR7⁻ NK cells). Activation by the local cytokine environment will influence CD27 expression. (B) All NK cells may develop from a CD27⁺ NK cell precursor. Interactions with its ligand, CD70, will downmodulate CD27. (C) CD27⁻ and CD27⁺ NK cell subsets may be derived from different precursor cells. CD27⁻ NK cells remain in the periphery, whereas CD27⁺ NK cells migrate to lymph nodes. Within the lymph nodes, CD27⁺ NK cells may interact with CD70 provided by activated dendritic cells or B cells resulting in downmodulation CD27.
The interaction between CD27 and CD70 has been shown to promote NK cell activation both in vitro and in vivo [72-74]. Still, the lack of murine markers of NK cell subsets hampers in vivo studies on the generation, maintenance and migratory properties of these NK cell subsets [66]. The potential use of CD27 as a marker of NK cell subsets enables in vivo studies in mouse models and will greatly improve our knowledge on the generation, differentiation and migration of NK cell subsets.

**Concluding remarks**

Immunocompromised children are at risk of developing severe complications on primary, secondary and reactivating herpesvirus infections. Still, seemingly healthy children may die from these infections, as we demonstrated in this thesis for primary VZV infection. Large-scale vaccination may protect all children from developing severe courses of infection. This strategy is assumed to be cost-effective and varicella vaccination has been implemented in regular childhood vaccination schemes in the USA and Japan. The ultimate goal of VZV vaccination is abandoning the virus from the community, as has been achieved for smallpox. We (chapter 5) and others propose that maintenance of immunological memory is dependent on regular antigen-specific boosting. Large-scale vaccination will significantly reduce the frequency of natural infections [75]. Although vaccinated children are protected from developing complicated courses of infection, other generations which were infected up to decades ago with wild-type virus, will not be re-exposed to the virus anymore. Since virus-specific memory may not only be crucial for protection on re-exposure, but also for controlling latent infection, we predict that this vaccination strategy may lead to an increase in the number of herpes zoster cases in the older individuals. Repeated vaccination of all individuals may be necessary, regardless whether memory responses were induced by vaccination or infection with the wild-type virus (Figure 2).

![Figure 2 Consequences of large-scale varicella vaccination.](image)

**Figure 2 Consequences of large-scale varicella vaccination.** (1) Large-scale varicella vaccination will protect individuals from developing varicella upon exposure to VZV. At this moment, it has not been known how long this protective effect may last. The number of natural infections will be severely reduced. This leads to less spread of the virus within the community and thus previously infected individuals are not boosted by re-exposure. Since we believe that immunological memory is maintained by regular antigenic boosting, this may lead to a higher incidence of reactivating VZV which may lead to herpes zoster in the older individuals who were infected with the wild-type virus. (2) Therefore, in this vaccination strategy individuals who were infected decades ago by the wild-type virus should also receive the vaccine on a regular basis, to provide boosting of the memory response.

Furthermore, large-scale vaccination may shift the susceptibility from children to adults, whose symptoms are usually moderate to severe. Cost-effectiveness of large-scale varicella
vaccination will have to be re-analysed. In addition, abandoning the virus from the community will take a lot of effort and time and meanwhile previously vaccinated individuals will be exposed to the wild-type virus. It is therefore important to know how long the protective effect of vaccination is maintained. Limited available data on this subject show that although vaccine-induced immunity persists for 10-20 years, the effectiveness of the vaccine decreases over time [75,76]. We therefore propose to only vaccinate people at risk for developing severe courses of disease upon infection, under strict surveillance.

Furthermore, vaccines that protect individuals from developing EBV- and CMV-related disorders (a.o.) will have to be developed. This will lead to a marked reduction in the number of hospitalizations. However, as we showed in this thesis, we cannot predict in all cases which children are at risk of developing a complicated course of disease. Otherwise healthy children may develop life-threatening courses of varicella. General practitioners will have to recognize early symptoms of severe varicella and send these children to the hospital immediately. Upon admission, antiviral therapy should be initiated, concomitant with an extensive study on phenotype and function of NK cell and T cell subsets. When NK cells and/or primed CD8\(^+\) T cells cannot be detected in the circulation, IL-15 therapy might help these children to combat the virus.

In recent years, much effort has been put into the elucidation of the mechanism of activation of NK cells. Activation receptors such as NCRs have been identified. Many of these activation receptors are still orphan receptors. The discovery of their ligands may help us to explain why certain healthy children are unable to cope with their virus infection. NK cell activation may be diminished in these children because of the absence of the ligands for NK cell activation receptors. Alternatively, VZV may have developed strategies to circumvent killing of infected cells by NK cells as has been observed for murine CMV. Murine CMV gp40 downregulates H-60, a high-affinity ligand for NKG2D receptors, and thereby inhibits NK cell activation [77].

At this point, CD8\(^+\) T cell responses to VZV cannot be studied properly. The analysis of virus-specific CD8\(^+\) T cells requires identification of immunodominant peptides. These peptides have been identified for CMV and EBV, but not for VZV. A strategy to determine the sequence of the peptides that can be used to detect virus-specific CD8\(^+\) T cells has been described by Kern et al. [78]. Overlapping multimers are generated from immunodominant proteins. These peptides are then pooled and examined for their ability to induce IFN-\(\gamma\) by CD8\(^+\) T cells. This "checkerboard analysis" is expensive and we were unable to perform such a strategy in our setting. Proteins of interest for such an analysis are (in case of VZV) IE62 [79], IE63 [80], and gE [81]. Recently, an attempt to identify epitopes by this laborious strategy was made by the group of Ann Arvin and they identified an HLA-A*0201-restricted immunodominant epitope derived from IE62 [82]. However, data presented in the paper were questionable and in our hands the peptide could not be used for detection of VZV-specific CD8\(^+\) T cells in HLA-A2\(^*\) individuals.

From a purely clinical point of view, the ability to investigate VZV-specific CD8\(^+\) T cells will provide more insight into the exact mechanism of VZV-specific immunity and may answer questions such as why certain seemingly healthy children develop life-threatening courses of varicella. Hopefully, the scientific community jointly with pharmaceutical industry will recognize the need for identification of CD8\(^+\) and CD4\(^+\) T cell epitopes in order to develop DNA vaccines against the various herpesviruses that can be safely administered to immunocompromised patients, to prevent serious courses of disease on infection.

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References


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