The role of cytomegalovirus infection in the induction of type 1 diabetes

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Chapter 9

Summary

Type 1 diabetes (T1D) is an autoimmune disorder which results from the selective destruction of the insulin-producing islet β-cells. Histologically, the destruction is characterized by insulitis, an inflammatory infiltration of the pancreatic islets, containing large numbers of mononuclear cells including $CD8^+$ T cells. Clinical manifestation of the T1D usually occurs within the first two decades of life. A strong rise has been reported during the last decades of the 20th century in Western countries. Genetic factors are implicated in the pathogenesis of T1D, however also non-genetic, environmental factors seem to be required. Besides dietary components, viral infections are supposed to be implicated in the pathogenesis of T1D (Chapter 1). One of the candidate viruses is cytomegalovirus (CMV), a double strains DNA virus and member of the herpesvirusfamily. Although several studies show an association between CMV infection and T1D, a causal relationship has not yet been found. However, we found a causative relationship between rat CMV (RCMV) infection and T1D in the BB rat, a well-established animal model for T1D. The aim of this thesis was to analyze the effect of CMV infection on the development of T1D and to elucidate the underlying pathogenetic mechanism. In chapter 2-5 several aspects are discussed concerning the underlying mechanism of the RCMV-induced accelerating effect on diabetes in BB-DP rats. In chapter 6-7 the effect of RCMV infection is analyzed on respectively cell-mediated immunity in BB-rats and alloreactivity in a ratmodel for chronical rejection after lung transplantation. Finally, chapter 8 covers the general discussion and chapter 9 the summary, both for experts and non-experts.

In **Chapter** 1, an overview is given of the viral infections implicated in human T1D including enterovirus, rubella virus, mumps virus, rotavirus and CMV. Possible cellular and molecular mechanisms are discussed by which these viruses trigger β-cell specific autoimmunity leading to T1D based on human studies as well as studies on spontaneous or induced animal models of T1D. In the introduction to the experimental work some characteristics of CMV are discussed such as structure, transmission, tissue tropism, cell entry and replication. At the end, the aim of this thesis was discussed.

In **Chaper 2**, the effect of infection with rat specific CMV (*i.e.* RCMV) was investigated on the development of T1D in the diabetes-prone (BB-DP) rat. Infection with RCMV (i.p. in accordance to the generally used infection protocol) at the age of 35 days resulted in significant acceleration of diabetes onset with 2-3 weeks. In order to improve our understanding of the underlying mechanism, the presence of RCMV-infected cells was determined in the pancreas, isolated islets and other organs one week after infection. RCMV-infected cells were only occasionally found within the pancreatic islets, arguing against a lytic infection of the pancreatic β-cells as a possible mechanism by which RCMV induces accelerated

diabetes. However, a selective accumulation of RCMV-infected cells (identified as macrophages) as well as uninfected MΦ was observed within the interlobular septa in the exocrine tissue of the pancreas one week after infection. This accumulation was selective, since RCMV-infected cells were besides pancreas, only detected in peritoneal cavity-associated tissues such as omentum and parathymic lymph nodes. Concomitantly, protein levels of the chemoattractant MCP-1 were increased both in serum and pancreas preceding the development of insulitis which was accelerated. These findings suggest that RCMV infection induces a selective accumulation of MΦ within the pancreas, which is accompanied and possibly caused by an enhanced MCP-1 production in the pancreas. As a result a local pro-inflammatory environment develops, which is most likely the key factor involved in the acceleration of insulitis and eventually diabetes in RCMV-infected BB-DP rats.

In **Chapter 3**, the role of pMΦ was discussed in our rat model of RCMV-enhanced diabetes. After a rapid influx of MΦ in the peritoneal cavity 1 day p.i., numbers of MΦ decreased and stabilized during the next 6 days. Depletion of pMΦ shortly after infection (day 1-3) resulted in abrogation of the RCMV-induced accelerating effect on diabetes suggesting that pMΦ play an essential role in acceleration of diabetogenesis. However, depletion at day 6-8 did not have any effect on the accelerating effect, suggesting that the "effector" pMΦ had already moved away from the peritoneal cavity within the period till 6 days p.i. Since an accumulation of MΦ was observed in the pancreas one week after infection, we hypothesized that MΦ start to migrate from the peritoneal cavity (site of infection) toward the pancreas after RCMV infection. In **Chapter 4** we determined the kinetics of macrophage infiltration (both RCMV-infected and uninfected) into the pancreas within one week after infection using immunohistochemistry. An influx of MΦ was observed as rapid as 1 day p.i., followed by a slight further increase in cell numbers during the next 6 days. Considerable RCMV-infected MΦ were detected from 3 days p.i. with peak numbers 5 days p.i. To test whether pMΦ migrate selectively from the peritoneal cavity to the pancreas after RCMV infection, pMΦ were selectively labeled with the dye PKH26 *in vivo* and several tissues were analyzed for the presence of $PKH26^+$ cells. $PKH26^+$ peritoneal cavity-derived MΦ were indeed observed in the pancreas as rapid as 1 day p.i. 7 days p.i., only a few $PKH26^+$ MΦ were detected, suggesting that after an initial influx of $PKH26^+$ peritoneal cavity-derived macrophages, also blood-derived MΦ are attracted. Pancreatic $PKH26^+$ MΦ expressed viral genes, suggesting that these cells play a role in viral dissemination from the peritoneal cavity to the pancreas. This cell recruitment was selectively directed toward the pancreas since $PKH26^+$ peritoneal cavity-derived MΦ were further only detected in peritoneal cavity associated tissues, as omentum and parathymic LN, occasionally in the liver and not in the peripancreatic LN,

cervical LN, mesenteric LN, salivary glands, lungs and spleen. The initial trigger for selective migration of pMΦ from the peritoneal cavity to the pancreas may be increased intrinsic expression of pancreatic MCP-1 in young BB-DP rats (compared with WAG/Rij rats, also RT1u).

In **Chapter 5**, we investigated if pMΦ indeed play an essential role in accelerating diabetogenesis. Therefore, we adoptively transferred pMΦ from RCMV-infected rats to naive BB-DP recipients and found that they were able to accelerate diabetes onset, indicating their essential role. Since several viruses have been shown to activate innate immunity via binding to Toll-like receptors (TLRs) resulting in inflammatory responses, we investigated the role of TLRs in the activation and recruitment of MΦ in our model for CMV-enhanced diabetes. BB-DP pMΦ expressed TLRs: TLR1-4, TLR6-7 and TLR13. RCMV infection of BB-DP rats increased expression levels of the chemoattractant MCP-1 and its receptor CCR2 in pMΦ 1 day p.i., suggesting that these cell become activated after RCMV infection. Incubation of freshly isolated BB-DP splenocytes with RCMV or TLR ligands induced MCP-1 expression *in vitro*. Incubation of splenocytes enriched for MΦ with RCMV or the TLR4 ligand LPS *in vitro*, resulted in downregulation of IRAK-3 (M) expression, a negative regulator of TLR signalling, suggesting that RCMV induces a LPS-like response. Together, these findings suggest that RCMV activates spleen-derived MΦ via TLR triggering resulting in induction of MCP-1. If RCMV activates pMΦ also via TLRs is likely, however, whether pMΦ are indeed activated via RCMV-mediated TLR triggering is currently under investigation.

In **Chapter 6** we studied the effect of RCMV infection on the cellular immunity in BB rats. The percentage of $CD8^+$ T-cells was slightly increased *in vivo* both in spleen and periperhal blood. Stimulation of RCMV-primed splenocytes with RCMV-infected fibroblasts *in vitro*, resulted in a strong proliferative response and increased blast transformation, which was RCMV-specific since stimulation with non-infected fibroblasts did not induce such a response. The $CD4^+/CD8^+$ T-cell ratio was reduced especially within the blastoid T cells (*vs.* ConA stimulation), suggesting a preferential expansion of $CD8^+$ T cell blasts. Indeed, $CD8^+$ T cells showed increased expression of the activation marker CD25 (IL-2r-αchain) and the pro-inflammatory adhesion molecules CD44h and LFA-1, indicating increased activation after stimulation with RCMV-infected fibroblasts *in vitro*. Percentages of Vβ-TCR percentages did not change after RCMV infection or RCMV-restimulation. Analysis of CFSE-dilution revealed that the strong RCMV-induced proliferative response had a polyclonal character and enhanced rounds of cell division were detected. These findings suggest that RCMV does not act as a superantigen, but rather induces (bystander) activation and proliferation of splenic T cells in a

polyclonal fashion. Probably also autoreactive T cells are activated and expanded, which may be involved in triggering autoimmunity *in vivo*.

In **Chapter 7** the hypothesis was tested that CMV infection could enhance rejection of lung grafts through generation of immune effector cells during CMV infection and immunosuppression. RCMV infection induced an increase in the number of NK cells and NK T cells 14 days after transplantation despite the use of cyclosporine-A (CsA) as immunosuppressive drug. Proliferative capacity of RCMV-primed splenocytes was increased after stimulation with both RCMV-infected syngeneic as well as infected allogeneic fibroblasts both in transplanted as well as non-transplanted rats. CsA-treatment of non-transplanted rats induced even higher proliferative reponses of RCMV-primed splenocytes against RCMV-infected allogeneic and syngeneic fibroblasts. These results suggest that RCMV infection of tissue cells (*i.e.* fibroblasts) either of donor or recipient origin enhances the recipient's cell mediated immune response even under CsA regimen. These proliferative responses were absent in RCMV-infected, transplanted rats treated with CsA, however, an increased infiltration of $CD8^+$ T cells was detected in the lung grafts of these rats, suggesting infiltration of these cells into the inflamed lung. RCMV infection did not affect intragraft gene expression levels of IFN-γ, IL-2, IL-10, IL-4 and TGF-β.

In **Chapter 8** the findings described in this thesis are discussed and integration of the different aspects led to a hypothetical mechanism by which RCMV induces acceleration of diabetes in BB-DP rats. Furthermore, different factors are discussed such as nature of the CMV, viral load, age of infection and encounter of infections during life time, which are proposed precipitating factors involved in initiation or acceleration of diabetogenesis in genetically susceptible individuals.