Targeting and exploiting cytomegalovirus for vaccine development

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Chapter 7

General Discussion

GENERAL DISCUSSION

T cells are essential components of naturally acquired protective immune responses in many diseases. Although various ways of inducing potent T cell response by vaccination have been assessed, the majority result in low level, non-protective immune responses. Vaccine development has broadened and currently vaccine platforms range from replication-deficient (attenuated) or killed micro-organisms to viral vectored vaccines and more recently to protein subunit vaccines. Many highly effective vaccines designed to induce protective $CD4^+$ and/or $CD8^+$ T cells against chronic infections and cancer have been developed. Although their precise mechanisms of protection depend on the complexity and type of antigen, the quality of the vaccine-induced T cell response is gaining increasing attention.

In this doctoral thesis, the SLP T cell-based vaccination strategy alone or in combination with agonistic antibodies triggering the costimulatory TNFR superfamily member OX40 were tested for their capacity to induce potent $CD4^+$ and $CD8^+$ T cell responses and to confer protection against lytic MCMV infection. Vaccination with MHC class I- and II-restricted MCMV epitope containing SLPs successfully eliminated virus spread in naïve mice but failed to boost host's antiviral immune responses and treat established MCMV infection. Direct correlates of protection were thoroughly investigated. Another vaccination model tested was the MCMV-based vector vaccine against virus-induced cancer (HPV). Injection of the MCMV vectored vaccine expressing an immunodominant MHC class-I restricted HPV E7 epitope led to long term protection against tumor outgrowth in naïve hosts but exhibited limited efficacy in hosts with a strong pre-existing immunity to MCMV. To optimise both vaccination strategies, we explored essential components required to shape protective immune responses and ways to subvert existing immune responses and improve immune recognition.

COMBINATION IMMUNOTHERAPY AGAINST MCMV

Several approaches can be used to improve immunity to MCMV-associated disease. Concomitant stimulation of $CD8^+$ and $CD4^+$ T cell responses via MHC class I and II epitope-containing SLPs, respectively, and augmentation of costimulatory signals mediated by the TNFR family member OX40 are very promising tools, which will be extensively discussed below.

Maximum virus control through activation of $CD4^+$ and $CD8^+$ T cells

The last decades, numerous immunotherapeutic platforms mainly focused at the induction of neutralizing antibodies have been tested against CMV. The majority of these vaccines exhibited low or short-term efficacy [1]. Whether sterile immunity against CMV is an achievable goal remains questionable mainly due to the numerous immune evasion mechanisms exploited by the virus. T cell-based vaccines designed to

induce $CD4^+$ or/and $CD8^+$ T cell responses is a rapidly growing field. Many studies have demonstrated the importance of T cell immunity against CMV. There is solid evidence that $CD4^+$ T cells are crucial for controlling CMV replication during the acute phase of the infection while $CD8^+$ T cells play an essential role during latency and virus reactivation [2-4]. SLP T cell based immunotherapy is a safe and well-explored vaccination platform, which results in the activation of $CD4^+$ and $CD8^+$ T cells and has shown remarkable potency to treat HPV associated disease in both preclinical and clinical studies [5,6].

In **chapter 2,** MHC class I epitope containing SLPs along with TLR9 agonists were tested in a prime-boost vaccination setting in various mouse strains for their ability to contain high load systemic MCMV infection. Vaccination led to the induction of vigorous and poly-functional (IFN-γ^+/ TNF^+/ IL-2^+) cytotoxic $CD8^+$ T cell subsets mediating potent and long-term protective immunity against MCMV infection. Vaccine-induced $CD8^+$ T cells slowly converted to a unique memory T cell subject sharing features from both effector and central memory T cells while their functionality was significantly improved throughout time. Notably, the size of each distinct SLP vaccine-induced $CD8^+$ T cell response was found unrelated to the functional avidity and proportional to the naïve T cell precursor frequency. This finding suggests that T cell precursor frequencies may be considered as a powerful model to predict the subsequent size of the T cell response induced upon peptide vaccination. In shared antigen or neo-antigen cancer vaccine trials *in vitro* stimulation with the targeted antigen is used to provide insight into the patient's immune system ability to respond to potential vaccination [7,8]. However, determination of the targeted antigen/peptide precursor frequency will possibly ease classic *in vitro* antigen selection processes and may form an important parameter to be considered in immunotherapeutic strategies that their efficacy is based on the selection of antigens.

Despite the importance of $CD4^+$ T cells in the immune system, the role of CMV-specific $CD4^+$ T cell responses in CMV infection has not been completely understood. $CD4^+$ T cell ''help" is likely to be crucial for increasing the effectiveness of candidate vaccines for CMV. Therefore, in **chapter 3** the efficacy of MCMV-specific $CD4^+$ T cells to control MCMV was studied using a vaccine that comprises MHC class II epitopes. In addition, the capacity of the $CD4^+$ T cell responses to enhance the efficacy of a MCMV-specific $CD8^+$ T cell response was studied by simultaneous injection of MHC class I and II epitope SLPs. $CD4^+$ T cells induced after vaccination with various MHC class II SLPs and OX40 ligation elicited broad antiviral Th1 cytokine responses and showed direct antiviral function against MCMV infection. Interestingly, vaccine induced $CD4^+$ T cell responses conferred moderate protection against lytic MCMV infection in both lymphoid and non-lymphoid organ tissues. These findings advance the findings of other reports showing direct antiviral effector function of CMV specific $CD4^+$ T cells in both mice and human [2,9-12].

Moreover, inclusion of $CD4^+$ T cell ''help" during vaccination with a combination of MHC class I SLP vaccines significantly enhanced $CD8^+$ T cell expansion and remarkably

improved the overall prophylactic vaccine efficacy. Notably, $CD4^+$ T cell signals increased priming of vaccine-evoked $CD8^+$ T cells suggesting a direct synergy between these T cell subsets. The concept that $CD4^+$ T cell ''help" to license DC for proper $CD8^+$ CTL priming must be antigen specific has long been concluded [13-16]. Thus, MHC class II epitopes are likely to add value if included in the design of epitope-based vaccines against CMV. Consequently, in **chapter 5** a plethora of highly immunogenic IE2 MHC class II restricted T cell epitopes where identified following traditional *in silico* screening methods. Whereas the T cell response to the IE1 HCMV protein is dominated by MHC class I T cell responses, limited $CD8^+$ T cell reactivity was measured against the IE2 protein. Since T cell responses to IE antigens predominate the lytic phase of the CMV infection, a vaccine formulation that will comprise IE1-specific MHC class I epitopes and IE2-specific MHC class II epitopes targeting immune dominant $CD8^+$ and $CD4^+$ T cell viral regions respectively is possible to act synergistically and inhibit the establishment of CMV latency.

The need for enforced OX40 co-stimulation

Therapeutic targeting of immune check point inhibitors (PD-1/ PD-L1 and CTLA-4) or costimulatory receptors (41BB/ 41BBL and OX40/ OX40L) has been beneficial against many chronic viral infections and cancer [17-24]. Costimulatory molecules provide critical interactions for inducing and maintaining adaptive immune response against CMV. Antibodies can specifically bind to costimulatory receptors and boost or inhibit an immune response.

In **chapter 3** agonistic OX40 antibodies in combination with various MHC class I and MHC class II epitope containing SLPs of MCMV-encoded antigens were tested in a prime-boost vaccination schedule. OX40 ligation synergized with the SLP-based vaccines and empowered overall vaccine efficacy but did not exhibit any therapeutic or prophylactic reaction when provided as monotherapy. Enforced OX40 activation strongly increased the number of-vaccine-induced $CD4^+$ T cell and $CD8^+$ T cell responses, especially when provided during booster vaccination. The effect of OX40 triggering was more pronounced on $CD4^+$ T cells presumably due to the higher expression of the OX40 molecule on this T cell subset [25,26]. OX40 mediated signals were not short lived or limited on effector T cells but influenced also memory T cell formation. Specifically, memory vaccine-derived $CD8^+$ T cells treated with OX40 agonists during booster SLP vaccination exhibited improved functionality, survival and secondary clonal expansion compared to the untreated subjects.

Interestingly, the timing and the number of doses OX40 agonistic antibody was administered regulated both the size and the quality of the subsequent vaccine-mediated T cell response. Specially, when agonistic OX40 antibody was provided during both prime and booster SLP vaccination all the positive effects of the OX40 triggering on T cell induction were diminished or utterly lost. A possible explanation

for this outcome is that short time intervals between the two vaccinations did not allow adequate memory T cell development and upon secondary OX40 stimulation apoptotic cell death events were dramatically accelerated. T cell susceptibility to activation-induced cell death (AICD) can occur in a cell-autonomous manner and is regulated by previous T cell activation history and the stage of the T cell maturity [27]. Repeated TCR activation and co-stimulation may promote the Fas/CD95 pro-apoptotic pathway whereas correct timing of co-stimulation can promote the Bcl-2 anti-apoptotic pathway [28]. Consistent with this, upregulation of the Bcl-2 molecule, known as a target of OX40, was measured when agonistic anti-OX40 antibody was provided only during booster vaccination and correlated with prolong T cell survival. In contrast, repeated enforced OX40 stimulation during both prime and booster SLP vaccination downregulated Ki67 and Bcl-2 expression leading to decreased T cell proliferation and survival. Additionally, it has been previously reported that Bcl-2 upregulation accompanied with increased expression of IL-2 inhibits AICD of previously activated T cells [28,29]. OX40 stimulation during booster SLP activation dramatically escalated autocrine IL-2 cytokine production levels and AICD events were delayed. Whether further treatment with IL-2 at the time of stimulation with OX40 agonists could overcome T cell susceptibility to AICD remains to be explored.

Another interesting observation was that IL-2 secretion was tightly regulated by OX40 costimulatory signals. Enforced OX40 stimulation mainly during booster SLP vaccination promoted ''Th1'' cytokine production by both $CD4^+$ and $CD8^+$ vaccine-specific T cell subsets. Strikingly, IL-2^+ $CD4^+$ and/or $CD8^+$ cytokine T cell responses were significantly bolstered during the effector phase of the vaccine response by OX40 stimulation and maintained at high levels throughout time. As a future development, it would be particularly intriguing to test whether IL-2 induction, currently provided in many combination immunotherapeutic settings through laborious adoptive transfers or high toxicity direct infusions could be complemented or even replaced by providing OX40 co-stimulation using agonistic antibodies.

Due to its capacity to regulate both $CD4^+$ and $CD8^+$ T cells, OX40 is considered a promising candidate in immunotherapy of persistent viral infections and cancer. However, there is preclinical evidence that anti-OX40 antibodies could induce off-target toxicity causing deleterious immunosuppressive side effects by promoting the accumulation of MDSCs and the production of Th2 cytokines leading to autoimmunity or inflammatory diseases [30-32]. In **chapter 3**, NK cell augmentation and Th2 cytokine activity associated with pro-inflammatory and autoimmune conditions were not triggered by OX40 stimulation. Presumably, combination immunotherapy with peptide-vaccines and low dose schedules of OX40 stimulation did not allow development of off target toxicity events. Moreover, agonistic OX40 antibody treatment has exhibited mild toxicity and no expansion of Tregs, when applied as monotherapy for the treatment of solid tumours [24], compared to the FDA approved CTLA-4 (ipilimumab) or PD-1/ PD-L1 mAb blockade therapies [33,34].

Despite all the positive effects of OX40 agonists it is unlikely that anti-OX40 as single agent will be sufficient to cure patients with different tumour types or viral infections. However, there is great promise that combination immunotherapy incorporating OX40 stimulation and vaccination may be able to increase efficacy. Finally, it would be particularly informative to investigate whether enforced OX40 triggering could complement the therapeutic activity of other costimulatory antibody (anti-4-1BB), checkpoint inhibitor (PD-1/ PD-L1/ CDLA-4) and conventional treatments (i.e. radiotherapy, chemotherapy, cytokine infusions). Encouraging results come from recent studies where anti-OX40 antibody treatment uniquely synergized with PD1-blockade to promote tumour regression in experimental models [34,35].

DIVERSITY IN SPECIFICITY

A valuable observation in **chapter 2** was that the prophylactic efficacy of the distinct MHC class I MCMV epitope containing SLP vaccines was remarkably potentiated when all individual SLPs were combined and administered as a mixture. The efficacy of the MHC class I SLP vaccines to protect against lytic MCMV infection was primarily driven by the breadth of the $CD8^+$ T cell responses rather than the magnitude of the response to all individual SLPs. Although the magnitude of the individual antigen-specific $CD8^+$ T cell responses was significantly reduced when provided within a mixture and the overall T cell response size was approximately similar to individual SLP vaccines, the ability to contain virus spread was drastically enhanced.

An interesting observation of the present work was the superior capacity of the M38 and m139 MCMV MHC class I epitope containing SLPs to reduce viral titers upon lytic MCMV challenge. M38 and m139 antigens elicit strong inflationary $CD8^+$ T cell responses during MCMV infection and presumably play an important role in regulating virus replication and persistence [36,37]. Conceivably, induction of strong $CD8^+$ T cell vaccine responses to the M38 and m139 viral antigens in combination with high expression of these antigens on MCMV-infected cells may explain the advanced efficacy of the M38 and m139 specific SLP vaccines in controlling virus spread.

Associations of potent T cell responses with increased breadth leading to decreased viremia or complete viral eradication has been observed in other chronic viral infections [38-41]. A possible explanation for this finding is that development of a robust cytotoxic T cell response with a diverse repertoire of specificities, targeting viral antigens which are expressed at different stages in the viral life cycle increases the likelihood of immune recognition of viral infected cells and the probability of virus dissemination control. Especially, there is evidence that potential encounter of viral infected cells with vaccine-induced T cells of different specificities boost cytokine-mediated direct cell killing and promote CTL cooperation events [42].

Although the exact mechanisms through which immune T cell repertoire diversity influences anti-viral immunity are not yet defined, it is important to consider induction

of a T cell response with increased breadth when designing prophylactic T cell-based vaccines against MCMV infection. Based on the results obtained from the chapter 2 the breadth of the vaccine response may be an important goal in vaccine formulations and a determinant of vaccine efficacy.

SHAKING DOWN THE RIGID WALLS OF PRE-EXISTING IMMUNITY

In many infection models, immunological memory to a viral vector is considered a hindrance for subsequent induction of cell-mediated and humoral immune responses. Various components of the immune system, including neutralizing antibodies, vector-specific T cells and type I IFN-activated NK cells contribute to seriously compromised immune responses against the delivered heterologous antigen [43]. Additionally, pre-existing immunity to a viral vector impacts viral vector's expression level, virus trafficking and alters homing patterns of vaccine induced T cells. Similarly, pre-existing immunity may modulate the magnitude, breadth and immune system's antibody and T cell response to the inserted antigen [44]. CMV-based vectors are endowed with the capacity to stimulate robust CTL and humoral immune responses and have shown unprecedented efficacy against persistent viral infections and cancer [45-47]. Due to the high prevalence rate of CMV in the population, pre-existing immune responses in the host may exist. However, compared to other viruses and traditional viral vector systems (i.e. adenoviruses, lentiviruses), CMV has the ability to re-infect or even superinfect the same host and initiate a second cycle of immune responses.

Thus, in **chapter 5**, the capacity of MCMV-based vector vaccines to induce antitumor responses against HPV-induced cancer was explored. Whether the presence of pre-existing immune responses to MCMV is detrimental or can augment response to the vectored antigen was also investigated. MCMV vectors encoded a dominant MHC class I HPV16 E7 epitope in either inflationary of non-inflationary MCMV epitope regions were tested for both prophylactic and therapeutic efficacy against HPV^+ tumors in mice. Notably, systemic or subcutaneous MCMV-vectored vaccine administration stimulated vigorous HPV-specific T cell responses recapitulating MCMV response pattern and provided complete and long term protection against tumor challenge in naïve mice.

The therapeutic testing of the MCMV vector vaccine prolonged survival of challenged mice but exhibited moderate immunogenicity when compared to mice with no immunological memory to the vector, eventually leading to minor therapeutic effect. Interestingly, the efficacy of the vaccine was associated with the level of pre-existing humoral immunity to MCMV. Specifically, the initial viral infection dose determined the magnitude of the subsequent antibody and T cell vaccine- specific response. Mice initially infected through the systemic or subcutaneous routes developed strong anti-viral immunity, which severely diminished viral vector's impeding vaccine efficacy. In addition, there is supporting evidence that the therapeutic efficacy of the CMV vectors expressing tumour or viral antigens is significantly attenuated when the host harbours

latent CMV [47]. We and others have previously reported that the initial viral inoculum dose impacts virus immune response [48,49]. Hence, we tested whether different levels of pre-existing immunity to MCMV can influence the subsequent MCMV-vector vaccine efficacy. Strikingly, when initial infection administered orally, weak T cell and antibody responses were elicited, which were significantly boosted following systemic or subcutaneous MCMV- vector vaccine administration. Consistently, the vaccine efficacy was escalated leading to almost complete eradication of tumor-bearing mice. In contrast, strong pre-existing immunity to MCMV was not surpassed by any of the conventional vaccine injection routes tested.

It is not surprising that MCMV – when applied as a vaccine vector containing tumor antigens – is not capable to overcome its own immunogenicity. Even therapeutic targeting of persistent MCMV infection using the strong SLP T cell-based vaccine concept in **appendix of chapter 3** was not capable to boost the considerable high levels of pre-existing virus-specific T cells present 8 weeks after viral infection. Whether the efficacy of the peptide vaccines in therapeutic settings would have been improved in case of low dose pre-exposure to the virus remains to be tested.

Our results suggest that T cell-based vaccine vectors must be designed to generate sufficient quantities of antigen and induce broad cytotoxic $CD4^+$ and $CD8^+$ T cell responses. Key outstanding research challenges in the use of CMV vectors are dealing with the diversity in the level of pre-existing immunity of pre-exposed individuals and in discovering ways to minimize this response to ensure that all CMV vectored vaccines will reach the threshold levels of protective immunity needed for efficacy. Several strategies, such as augmentation of viral vector dose, immunization route and timing are important factors to circumvent pre-existing immunity to CMV and need to be considered. Moreover, strategies to lower pre-existing immunity through dynamic targeting of critical genes/proteins for virus replication and latency are likely to be particularly informative. Importantly, deletion of virally encoded inhibitors of MHC class I antigen presentation may be essential for blocking establishment of persistent secondary infection or superinfection in CMV rhesus macaques [50]. Finally, the design of distinct CMV serotypes to overcome immunological memory might be a feasible future direction. Analysis of the components involved at the regulation of pre-existing MCMV immunity will help further improve the development of therapeutic CMV vaccines and vector delivery systems for animal and human use. [51]

CONCLUDING REMARKS

T cell mediated protection is multifaceted and driven by several factors. Despite differences among viral infectious diseases, common determinants of the vaccine efficacy is the magnitude, breadth, availability of co-stimulation, tissue location and functionality of $CD8^+$ T cells **(chapter 2, 3, 4 and 6)**. The role of $CD4^+$ T cell ''help" in providing direct effector function and regulating the magnitude of the $CD8^+$ T cell

response is crucial (**chapter 3**). Therapeutic interventions with MCMV vectors appear to be promising for chronic viral infections (**chapter 5**). A fundamental research question which the findings of this thesis put forward is whether other factors such as innate immunity and B cells are required for sufficient vaccine function and how these factors can be co-manipulated to optimize vaccination.

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