Chapter 9

General Discussion

Lack of tolerance against "self" antigens or "self" commensal bacteria leads to autoimmune and inflammatory disorders. Current treatment of those diseases is symptomatic, not specific and unfortunately often not effective. Recent advances in the fields of immunology, cell biology, genetics and bioinformatics led to identification of new therapeutic targets and tools for treatment of autoimmune and inflammatory disorders. One of these novel prevention and treatment strategies is induction of immune tolerance. This thesis focuses on inducing immune tolerance by regulatory T (Treg) cells generated *in vitro* by activation of naive T cells, or *in vivo* by adeno-associated virus (AAV)- mediated delivery of immunomodulatory peptides (Part I). We also explored novel methods for tolerance induction with the aim to prevent unwanted immune responses directed at AAV vector capsid or immunogenic transgene product in the setting of gene therapy (Part II).

Part I Summary

Treg cells are a cellular component of the immune system that is specialized in suppressing immune responses of effector cells. They can be divided into naturally occurring, thymus-derived Treg (nTreg) cells and induced Treg (iTreg) cells which develop outside the thymus under specific conditions. Treg cells are responsible for sustaining homeostasis of the immune system, and deficiency of Treg in the system is generally associated with severe inflammatory disease states. Treg can be induced ex vivo and have proven to be safe and efficient in clinical trials for graft versus host disease [1, 2]. Treg mediated immune tolerance may also be employed as a treatment for diseases with an autoimmune or inflammatory background [3-8]. We have studied the latter in this thesis and used as *in vivo* model of inflammatory and autoimmune diseases two murine models of Inflammatory Bowel Disease (IBD) (CD45RB transfer and trinitrobenzene sulfonate mouse models). These models serve as mouse analogs of two chronic inflammatory disorders in humans, Crohn's disease and ulcerative colitis. There is no definite, curative treatment available for those conditions and patients require lifelong symptomatic management.

Although the exact etiology of the IBD is unknown, it is thought to originate as a result of genetic and environmental factors that lead to inappropriate immune responses against dietary or bacterial flora antigens present in the gut lumen. Those unwanted immune responses are specifically associated with activation of Th17 or Th2 cells and the inability to shut down the resulting immune-mediated inflammation. Clearly, inflammatory control by regulatory T cell therapy is an attractive treatment strategy for these disease states. A major limitation to the use of nTreg cells is availability as they represent only a small percentage of the peripheral circulating $CD4^+$T cell population. In order to overcome this issue, several groups have developed various methods to expand nTreg *in vitro* while keeping their functionality. Generally, the technologies are complex, time-consuming and the plasticity of nTreg cells in artificial environment during *ex vivo* expansion may lead to loss of their suppressive activity. Additionally their relative mature stage of differentiation makes expansion *in vitro* a difficult process.

In **Chapter 2** we describe a new simple method to generate stable and functional human iTreg cells *in vitro*, providing a simple alternative to previously reported techniques. We applied this protocol to murine cells as reported in **Chapter 3** and demonstrated the functionality of the generated iTreg cells, *in vivo* by their potential to ameliorate the disease phenotype in a CD45RB transfer colitis mouse model. The iTreg cells can also be induced *in vivo* by a variety of immunomodulatory peptides such as for instance cationic host defense peptides that have been successfully employed for treatment of inflammatory and autoimmune diseases. Among those peptides are recently discovered regulatory T cell epitopes (Tregitopes) that are derived from immunoglobulin G (IgG). Tregitopes, which are stimulators of $CD4^+CD25^+Foxp3^+$ T regulatory cell (Treg) expansion. The anti-inflammatory potential of Tregitope 167 and Tregitope 289 has been previously reported [9- 11], but a limiting factor of this treatment is the achievement of stable, therapeutic levels of the immunomodulatory peptides. A solution to this problem could be a gene therapy approach that would provide stable peptide expression after the delivery of the gene that encodes the peptide of interest and the most promising vector that can provide long term expression of the delivered gene after single administra-

tion is adeno-associated virus (AAV). AAV-mediated gene therapy has been effective and safe in preclinical studies as well as in several clinical trials [12-15]. In **Chapter 4**, we report the development of an AAV-based approach to deliver the anti-inflammatory Tregitope 167 peptide. Tregitope 167 transgene was delivered intravenously by an AAV vector in the trinitrobenzene sulfonate (TNBS) mouse model of IBD and this resulted in decreased intestinal inflammation. Hence, tolerance induction using Treg might be a future prospect for inducing immune tolerance in autoimmune and inflammatory diseases.

Part II Summary

A main concern with AAV-based gene therapy is the presence of pre-existing neutralizing antibodies (NAB) against AAV due to naturally occurring asymptomatic infections with the wild type virus or due to prior treatment with an AAV vector. Those anti-AAV NAB can inhibit transduction upon first administration in case of pre-existing immunity or upon re-administration with the same AAV vector serotype [16-19]. As an alternative to repeated delivery of the same AAV serotype, cross-administration, which is the sequential use of different AAV serotypes, may be considered. AAV serotypes 5 and 1 have been shown to have no significant inhibitory cross-reaction. In **Chapter 6** we have demonstrated that AAV serotypes 5 and 1 can be used sequentially for re-administration in the liver with no significant inhibitory cross-reaction observed. A non-human primate experiment is in preparation to confirm the data obtained in mice.

Cross administration of different AAV serotypes for re-administration of therapeutic gene, might not always be feasible, as different AAV serotypes [17] have different tissue tropisms. Therefore, a careful selection of appropriate AAV serotypes is required when employing this approach for a specific target tissue.

Another option to avoid formation of NAB against AAV vectors would be modifying the AAV vector capsid to exclude viral epitopes which induce immune response upon presentation to the immune cells [20]. Finally, the most

common and widely applied approach to inhibit immune responses is use of immunosuppressive drugs [21]. Our group has investigated the influence of bortezomib and anti-CD20 alone or in combination therapy on NAB against AAV capsid formation (in press). We describe this approach in **Chapter 7**. This approach clearly reduced immune responses, but the effect was short-lived which indicates that in order to reach satisfying and long-term results extended treatment regimens will be necessary.

Another obstacle that needs to be overcome in AAV-based gene therapy is the appearance of immune responses against the expressed protein which might result in loss of therapeutic gene expression [22-26]. MicroRNA, mir-142-3p, which is specifically expressed in antigen presenting cells (APCs) may be used as a novel approach to avoid transgene directed immunogenicity. Incorporation of mir-142-3p target sequences within a transgene sequence has been shown to prevent of mRNA and protein expression in haematopoietic lineage cells, including APCs in both *in vitro* and *in vivo* setup [27]. The use of mir-142-3p target sequences prevented immune responses towards the transgene product in mice when a lentiviral vector was used for gene delivery targeting the liver [28, 29]. Furthermore, our group provided evidence that both humoral and cellular immune responses against the transgene product can be efficiently reduced by use of mir-142-3p target sequences in AAV-based intramuscular gene delivery and these experiments are summarized in **Chapter 8**.

Conclusions and future perspectives

The major achievements reported in this thesis are the identification of two novel approaches to generate regulatory T cells with the capacity to ameliorate inflammatory response and to restore immune tolerance. Additionally, in relation to AAV-mediated gene delivery approach to induce tolerance, we developed new strategies to prevent specific immune responses to the transgene product or to the adeno-associated virus (AAV) vector capsid. Initially, we provided the basis for further clinical development of cell therapies that involve Treg cells for the treatment of autoimmune and inflammatory diseases

by developing approaches to generate both in vitro and in vivo Treg cells. Next, we reported a functional approach to reduce the immune responses against the transgene product after intramuscular delivery by an AAV vector. This strategy could be applied in any AAV vector- based gene therapy targeting the muscle where there is a risk of immune responses against transgene product. We are currently pursuing further research to evaluate the impact of mir-142-3pT regulated AAV gene delivery on the normal miRNA profile in the muscle tissue.

We also investigated the feasibility of cross-administration of AAV vectors, as an approach to avoid the problem of formation of neutralizing antibodies (NAB) against AAV capsid following primary delivery. Such antibodies may interfere with AAV vector transduction upon re-administration of the same serotype. We showed that AAV5 and AAV1 could be sequentially delivered and the NAB against the capsids of those AAV vectors do not cross-react. Hence, this is an attractive approach for therapeutic protein re-administration. Our study was performed in a mouse model and should be confirmed in non-human primates before its possible translation to the human patients.

We subsequently studied the effect of immune suppressive regimens on neutralizing antibody (NAB) formation against the AAV capsid. Bortezomib and/or anti-CD20 treatment did not lower the anti-AAV NAB level to a value that would permit the re-administration of AAV vector. Therefore, there is a need for further studies that would include longer treatment time, dose-finding and the introduction of additional immunosuppressive therapeutics that would influence not only the B but also the T cell population.

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