The spleen as essential organ in the immune respons to encapsulated bacteria

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Immune responses to pneumococcal polysaccharides (PPS) derived from the capsule of *Streptococcus pneumoniae* show a completely different response pattern compared to protein antigens. IgM as well as IgG antibodies are rapidly induced but also decline quite fast [1-6]. The nature of the induced memory is still a mystery as secondary (boost) vaccination does not lead to increased antibody levels [6], but clinical protection does seem to be obtained [4;7;8]. Classical IgG switched memory cells are not induced but longevity of antibodies is thought to compensate for these cells [9]. The splenic marginal zone has been shown to play a vital role in the rapid response against polysaccharide antigens [10-14]. Children below the age of 2 years and patients receiving chemotherapy show a reduced anti-polysaccharide response. The explanation for this most likely is due to a non-functional marginal zone.

In this thesis, studies are presented, concerning the relation between the splenic marginal zone and anti-PPS antibody responses in the normal situation and when affected by chemotherapy. We have analyzed the development of the human spleen in relation to the immature anti-PPS response in infants. Because chemotherapy results in an increased risk for infections caused by *Streptococcus pneumoniae*, we investigated the effects of chemotherapy on B cell subpopulations like marginal zone B cells and the consequences on anti-PPS responses. Since vaccination is a good option to prevent infections by *Streptococcus pneumoniae*, we analyzed the characteristics of PPS vaccination in comparison with PCV vaccination. Finally, we investigated whether vaccination can be expected to contribute to a higher level of protection against infection with *Streptococcus pneumoniae* during and directly after chemotherapy.

The splenic marginal zone

The splenic marginal zone is a unique compartment, in its cellular and anatomical composition only found in the spleen [15-17]. It appears to be designed for the rapid induction of humoral immune responses [14;18;19]. In **chapter 2**, we extensively review the unique features that make the splenic marginal zone so well equipped for rapid induction of humoral immune responses. The key issue in the induction of rapid responses to novel blood-borne antigens is that naïve B cells can respond to antigens even with antigen receptors that are of low affinity. The capability of B cells to respond with low affinity antigen receptors enables a rapid response to a broad variety of antigens [18]. Recently it has been demonstrated that the antigen receptors of naïve marginal zone B cell are multi-reactive [20-22]. The splenic marginal zone is located centrally in the blood-flow and because of the presence of a sinus (at least in the rat) in which the splenic arterial blood-flow opens, the blood-flow is substantial reduced [23]. This enables an intimate contact between antigens and effector cells. In addition, B cells express surface molecules like CD21, CD81, CD19 and Leu-13 that enable a lowering of the threshold for activation [14;24-26]. All these features (multi-reactivity and lowering of threshold for activation in combination with the low blood-flow) make splenic marginal zone B cells one of the first cells to come in close contact with novel blood-borne antigens, capable of responding to these antigens. Marginal zone B cells express high levels of surface CD21 [27], which is able to crosslink with surface IgM and facilitates the binding of complement-opsonized antigens, especially C3d opsonized capsular PPS [28-30]. The capsular polysaccharide antigens of encapsulated bacteria are so-called T cell independent type 2 (TI-2) antigens and fail to stimulate MHC class II dependent T-cell help [31;32], indicating that another mechanism is involved in the response to TI-2 antigens. The splenic marginal zone provides an optimal microenvironment for rapid responses to TI-2 polysaccharide antigens.

Antigen specific B cells are induced to proliferate already within the first day of primary immunization with TI-2 antigens [33]. In T cell dependent (TD) responses, an interval of 72 hours is noted after primary immunization because the process of T cell priming is the rate

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limiting step in TD responses [33;34]. Initiation of rapid responses to TI-2 antigens is confined to the marginal zone but once activated, B cells do not have to be in a specific splenic microenvironment in order to enter cell cycle [33]. Marginal zone B cells that have engaged TI-2 antigens, migrate to the outer PALS probably because of the need for T cell derived factors [33]. Large clusters of antigen-specific blast are mostly seen in the outer PALS and in extra-follicular foci in the red pulp areas adjacent to the T cell zones [33;35] and are most likely responsible for the production of massive amounts of neutralizing antibodies in an early phase of infection. In addition, some activated marginal zone B cells will migrate to the germinal center, initiating a germinal center process but because of lack of direct T cell contact, these germinal center will not produce high affine, IgG switched memory B cells [36;37].

The TI-2 response is characterized by a rapid induction of IgM antibodies concurrent with the induction of IgG antibodies. Normally, for isotype switching of germinal center B cells, the presence of immunecomplexes is required [34]. These immunecomplexes are composed of specific antibodies and antigen. Because of the rapid induction of IgG titers, the switching of polysaccharide specific antibodies must be independent of immunecomplexes with specific immunoglobulins because at this time point specific immunoglobulins are not yet present. In chapter 2 we argued that this rapid isotype switching is facilitated by the high expression of CD21 on marginal zone B cells in combination with lowering of the threshold for activation and multi-reactivity. We propose that after the first contact, an alternative affinity maturation and isotype switching takes place that is enabled by polysaccharide-C3d complexes localized on germinal center FDC's without the presence of specific immunoglobulins. This explains the rapid induction of IgG switched antibodies. The rapid induced IgG's are most likely of only low affinity but B cells with higher affinity will be generated following further antigenic contact.

The marginal zone appears to be a compartment totally dedicated to rapid humoral responses to (blood-borne) antigens. Because of it's specific abilities and the central position in the blood stream, marginal zone B cells are crucial in the first line defense against life threatening infections by blood-borne organisms. Marginal zone B cells can use complement C3d as a component of the innate immune system to enable a quicker involvement of the generally slower but more specific acquired immune system.

Development of the spleen

Children below the age of 2 years have immature immune response characteristics against especially polysaccharide antigens, most likely because of an immature spleen. In **chapter 3** we further analyzed the development of the infant spleen.

In the past, it was demonstrated by immunohistochemistry that all cellular compartments of the spleen of children have completed their maturation to an adult type immunophenotype and morphology within the first five months except for the marginal zone [12]. The main characteristics of the infant MZ was the low or absent expression of CD21 (CR2) on the marginal zone B cells [12]. Because the humoral response to encapsulated bacteria is highly dependent on a functional marginal zone with high expression of CD21 [10;13;27;38], children below the age of 2 years are defective in initiating an adequate response to capsular polysaccharides derived from encapsulated bacteria [7;8;12;39].

The marginal zone of the human adult contains mostly memory B cells. This was demonstrated by two approaches. It was demonstrated by Vh gene analysis [40;41]; mutations in the Vh gene are a hallmark of memory B cells [42] and it was demonstrated by CD27 expression [43]; memory B cells are CD27 positive [44;45]. In chapter 3 we analyzed the presence of memory B cells in the infant marginal zone by staining the marginal zone B cells for the presence of CD27 expression.

In infant spleens below the age of 2 years, because of the absence of CD27 expression, the marginal zone B cells are supposed to be of naive phenotype [46]. Between the age of 2 to 5 years, the number of CD27 positive B cells increased to adult numbers around 5 years of age. The increasing number of memory B cells in the marginal zone most likely is driven by ongoing antigenic stimulation.
We speculate that the immaturity of the humoral anti-polysaccharide response in infants below the age of two years may have its effect on induction of the humoral response as well as formation of memory. It seems that the infant spleen is fully matured and functional at 2 years of age and that memory B cells are capable of entering the marginal zone at this time point.

Evolutionary benefit because of the absent CD21 expression in early life?
Is there an evolutionary benefit of the absence of CD21 expression in the marginal zone of an infant below the age of 2 years? In the literature, there are some indications that the naive marginal zone B cells are involved in autoreactivity and the induction of tolerance [14;47-49]. Chen et al. demonstrated that autoreactive B cells in 'B cell activation factor from the TNF family' (BAFF) transgenic mice can colonize the marginal zone [20]. BAFF can prevent autoreactive B cells from going into apoptosis [50]. In BAFF transgenic mice, immature autoreactive B cells can be rescued by BAFF from negative selection. The mature autoreactive B cells skewed towards the marginal zone compartment [47]. Further, Fagarasan and Honjo have constructed a model for regulation of the autoreactive B cell response [51]. When autoreactive B cells accidentally become activated, this signal is most often suboptimal because of weak cross-reactivity, absence of opsonization or limited amounts of stimulants. This suboptimal signal will induce PD-1 expression. These PD-1 positive B cells can survive and enter the circulation. The ligand of PD-1 is expressed on various organs and ligation of PD-1 results in the suppression and elimination of activated autoreactive B cells, preventing activation of autoreactive B cells in the periphery [51].
The absence of CD21 expression on marginal zone B cells may be necessary to prevent autoreactive marginal zone B cells from reacting easily to autoantigens with subsequent differentiation to antibody secreting plasma cells. This mechanism of preventing marginal zone B cells to react to autoantigens may be essential to allow clearing of self-antigens from the circulation and induction of tolerance in an early period of life despite the fact that this is at cost of an increased risk for blood-borne infections.

Functional aspects of anti-polysaccharide responses in the immunocompromised host
Suppression of the immune system in children with malignant diseases is a main problem [52-54]. The cause of suppression is mostly twofold, on one hand by the immune de-regulation as a consequence of the disease and on the other hand by the use of chemotherapeutic agents. Infections with encapsulated bacteria appear to be responsible for a higher morbidity and mortality in patients treated with chemotherapy [54-56]. The neutropenic and granulocytopenic effects of chemotherapy are best known but also the lymphoid system can be heavily affected. Initially we looked at the effects of a single dose of a single cytostatic agent on B cell subpopulations in the spleen, blood, and bone marrow (**chapter 4**). We used the cytostatic agents cyclophosphamide, cisplatin, and methotrexate because these agents are known for affecting the humoral immune system [57-60]. Rats were treated with a single dose of a single cytostatic agent and sacrificed at different time points after treatment. We demonstrated that especially the rapidly dividing immature cells were highly sensitive to the tested cytostatic agents directly after treatment. The mature B cell populations appeared to be relatively unaffected. Analyzing the B

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Subsequently, in **chapter 5** we analyzed the effects of a reduced marginal zone population on the capacity of rats to initiate an immune response to PPS by challenging with antigens at different time points after chemotherapy. Chemotherapy was similar to the therapy used in chapter 4. We used two vaccines, the PPS vaccine Pneumovax® (TI-2 antigens), and the tetanus toxoid vaccine Tetavax® (TD antigens). We used vaccines as antigenic challenge to test the immune response capacity after chemotherapy and to mimic a blood-borne bacterial infection. The immune response capacity to PPS was reduced directly after chemotherapy. In contrast, the response to the TD antigen tetanus toxoid appeared relatively unaffected. When we analyzed the immune response capacity at a longer period (day 24) after treatment, we found normal immune responses to PPS as well as tetanus toxoid. These findings seem to be in contrast to our finding in chapter 4. Directly after chemotherapy, when only immature cells are affected and marginal zone B cells relative unaffected, immune responses to PPS are reduced while after a certain time period, when the marginal zone B cell population is (still) reduced, the immune response to PPS is normal. Cytostatic agents mostly affect activated, rapidly dividing cells [58;61]. Most marginal zone B cells, which are activated by PPS, will migrate to the outer PALS and red pulp to undergo clonal expansion [62]. During this expansion, they are possibly inhibited in their activity because of the cytostatic agents. At a longer period after chemotherapy, when the cytostatic agents are cleared from the circulation, the reduced number of marginal zone B cells that survived are still capable of initiating a response to the PPS, now without killing of the activated cells.

Multi-dose combination chemotherapy in the rat

In chapters 4 and 5 we used a single dose of a single agent to analyze the effects of chemotherapy on the humoral immune system. To better match the human situation in which extensive multi-dose combination chemotherapy is used, we designed a multi-dose combination chemotherapy for rats based on our own experience and an extensive literature study (**chapter 6**). This multi-dose combination chemotherapy consisted of 4 consecutive days of a single injection of cyclophosphamide followed by 4 consecutive days of a single injection of cisplatin. Similar to the studies in chapters 4 and 5, we first analyzed the effects of multi-dose combination chemotherapy on B cell subpopulations followed by the analysis of the immune response capacity to PPS and tetanus toxoid. Directly after finishing therapy, B cell populations were severely reduced. B cell populations appeared to be recovered after 25 days. We did not find any essential effect on other cell populations like T cells, macrophages, and monocytes. Directly after multi-dose combination chemotherapy, the IgG but not the IgM response to PPS was significantly reduced compared to control vaccinated rats. At 25 days after treatment, immune responses appeared normal again. The immune response to tetanus toxoid was relative unaffected.

Data regarding the effects of chemotherapy on human splenic B cell subpopulations and immune response capacity are very scarce and can only be done with *in vitro* experiments. Human peripheral blood lymphocytes have been used extensively in stimulation experiments with PPS and demonstrated that it is possible to stimulate peripheral blood B lymphocytes with PPS to induce specific antibody production [63-66]. *In vitro* experiments to determine the effects of cytostatic agents have been done with human peripheral blood lymphocytes but never with human spleen cells [67-71]. Our results obtained from the *in vitro* experiments (**chapter 6a**)

support our animal experimental findings that especially the marginal zone B cell is vulnerable for cytostatic agents.
From the studies described in chapter 4, 5, 6, and 6a we conclude that immature B cell populations are the most severe affected by chemotherapy but that these cell populations also rapidly recovered. The mature cell population, including the marginal zone B cell population is less directly affected but declines in numbers well after finishing chemotherapy. This is explained by the fact that replenishing of the mature B cell population is delayed because of the strong reduction of immature progenitor B cells. The recovery of the mature B cell populations like the marginal zone B cells takes a substantial amount of time and extends beyond the period of bone marrow suppression. In human studies, only little information is present regarding the slow recovery of marginal zone B cells after chemotherapy and total body irradiation [72]. By analyzing spleen sections of patients treated with immunosuppressive agents, it was demonstrated that the recovery of the marginal zone was poor up to 10 months after treatment and results in a functional asplenia for a considerable period [72].
From our results, the immune responses to TD antigens like tetanus toxoid appeared to be relative unaffected while the polysaccharide response showed reduced antibody titers after chemotherapy. However, the response to PPS was not completely absent indicating that even a few marginal zone B cells are able to induce a response to PPS. Although this response is lower compared to non-treated animals, antibody fold increases are still 4 times or more and it has been shown that an antibody increase of 3 to 4 times can be considered clinically relevant [8]. Still, patients treated with severe multi-dose combination chemotherapy are at increased risk for infections with encapsulated bacteria, not only during but also after chemotherapy [52;54;55;73-77]. However, not only the level of the antibody titer is relevant for protection but also the kinetics of antibody production in combination with the affinity of the produced antibodies is crucial, especially in case of rapidly spreading infections by encapsulated bacteria. An additional problem with the depletion of marginal zone B cells during chemotherapy is the loss of memory because marginal zone B cells are in human adults mostly memory cells. Memory B cells present in the marginal zone are of the IgM phenotype and slightly activated, hence, they are most likely more sensitive to chemotherapy compared to classical IgG switched memory B cells. The depletion of marginal zone B cells does not only result in the reduction of a B cell population capable of responding fast and efficient to encapsulated bacteria but also results in the loss of IgM memory B cells.

Immune responses to pneumococcal polysaccharide vaccine

Vaccination with PPS can prevent infections by *Streptococcus pneumoniae* but the immune responses mechanism to PPS vaccines are still poorly understood although vaccination is widely recommended in risk groups like elderly and immune compromised patients. Especially the induction of memory after vaccination with PPS is a main issue [2;7;8;78]. IgM as well as IgG antibody titers rapidly increase to peak values after primary vaccination but also decline quite rapidly to pre-vaccination values and classical IgG switched memory B cells are not found [79]. Vaccination with protein antigens results in a slow induction of IgG titers, which can be boosted with a secondary vaccination to higher titers. In contrast, PPS show a complete lack of response during secondary vaccination when humans are vaccinated within 3 to 5 years after primary vaccination [4-6;80]. The absence of a secondary response has been called immune paralysis [80]. In **chapter 7** we further analyzed the phenomenon of immune paralysis in rats. We used three different doses for primary vaccination because in mice it was demonstrated that the absence of a secondary response is dependent on the dose used in the primary vaccination [80-82]. In rats, we

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also found an absence of a secondary response (within the range tested) but this was independent of the dose we used in the primary immunization. When a secondary vaccination was given at one month after primary vaccination, an almost complete absence of antibody induction was observed. At 3 to 6 months after primary vaccination, a secondary vaccination resulted in an increase in antibody titers although titers never exceeded titers obtained in the primary vaccination. Analyzing spleen sections of rats that received a secondary vaccination one month after the primary vaccination revealed a normal localization pattern of PPS in the spleen with additional localization of PPS in secondary follicles. This indicates that although PPS show normal localization, activated B cells are partly "paralyzed" in their further activation and production of high amounts of antibodies. Based on a series of classical experiments by Baker and Braley-Mullen, immune paralysis after primary vaccination most likely is due to activation of CD8+ suppressor T cells [80-83]. The direct localization of PPS in secondary follicles during secondary vaccination could indicate that after secondary vaccination, PPS can bind residual PPS specific antibodies generated in the primary response and that these immune complexes subsequently immediate localize in follicles. This could indicate that although antibody levels remain low upon secondary vaccination, the antibodies produced are of high affinity.
Although a secondary vaccine response is suppressed, primary vaccination with PPS has empirically been demonstrated to be protective. This indicated that, although the secondary response to the same purified polysaccharide serotype is suppressed, the secondary response to the complete bacteria with the same serotype is not suppressed. Two reasons for this apparent paradox can be postulated; first is that the purified polysaccharide is slightly different from the crude polysaccharide derived from the whole bacteria and that the activated suppressor T cells are highly specific. Second, other components of the whole bacteria may act as a sort of natural adjuvant or natural conjugate, overruling the suppressor T cell activity. Further research will be necessary for complete elucidation [84].

Immune responses to polysaccharide conjugate vaccine

Conjugation of PPS to proteins leads to a polysaccharide response with protein response characteristics [85]. A pneumococcal polysaccharide conjugate vaccine (PCV) induces memory similar to protein vaccination and has been demonstrated to be immunogenic in children below the age of two years [39]. The major drawback of PCV is the limited number of polysaccharide-conjugated serotypes [7]. A 7-valent conjugate vaccine is registered and used and an 11-valent vaccine is being developed. However, the non-conjugated polysaccharide vaccine contains the 23 most prevalent serotypes and a conjugate vaccine containing 23 serotypes will not be possible due to protein overload [7]. Hence, the current pneumococcal polysaccharide conjugate vaccine is not the ultimate pneumococcal vaccine. In **chapters 8** and **9,** we described the spleen and complement independency of the PCV. The response to plain PPS has extensively been demonstrated to depend on the presence of complement, especially fragment C3d [86;87]. In complement depleted rats, no localization of PPS on marginal zone B-cells was observed [38]. In contrast, complement depletion did not impair responses to PCV and localization patterns in the spleen were similar in complement depleted and control rats indicating that the response to the conjugate vaccine is independent on the presence of complement and thus likely independent of strongly CD21 positive marginal zone B cells.
Absence of a functional spleen results in an increased risk for infections caused by encapsulated bacteria [88;89]. The liver can partially take over the phagocytic functions of the spleen but not for the not or low opsonized PPS because of the higher velocity of the blood-flow through the liver [90]. Vaccination to prevent infections should be performed at least a few weeks before

splenectomy. Vaccination with PPS after splenectomy is no option because of the absence of initiation of a response leading to antibody induction after splenectomy. The polysaccharide conjugate vaccine in contrast, shows normal immunogenicity in splenectomized rats as would be expected for a "protein-like" response. So, extra-splenic lymphoid tissue is capable of generating a primary response to the polysaccharide conjugate vaccine. Although the primary response to PCV was delayed, the secondary response was similar to the secondary response in control rats. The PCV has as an advantage that it can induce antibody response in the absence of a functional spleen but the limited number of conjugated serotypes remains a serious problem.

In **chapter 10**, we examined whether vaccination before chemotherapy can contribute to a higher level of protection during or directly after chemotherapy as measured with an antigenic challenge directly after chemotherapy. After MTX treatment, but not CyPh treatment, we observed improved antibody responses similar to control vaccinated rats. PPS antigenic challenge directly after MTX treatment with primary vaccination before MTX treatment did result in antibody titers similar to control vaccinated rats, whereas without vaccination significantly lower antibody levels compared to control vaccinated rats are observed. This indicates that vaccination before chemotherapy contributes to a better response directly after chemotherapy. It can be conclude that after primary vaccination, (MTX resistant) memory is likely induced resulting in a secondary response, which is independent of the marginal zone.

Concluding remarks and perspectives

For the host, polysaccharide-encapsulated bacteria have a major disadvantage in the induction of a "normal" immune response because the capsular PPS cannot directly activate T cells. To circumvent this limitation, the immune response to PPS is initiated in the splenic marginal zone. Due to several specific characteristics and features, marginal zone B cells are capable of initiating a fast response with high quantities of neutralizing antibodies, IgM as well as IgG, without the necessity of direct T cell contact. Absence of a functional spleen thus leaves the host at risk for severe infections caused by polysaccharide-encapsulated bacteria.

Infections with *Streptococcus pneumoniae* can be prevented by vaccination with PPS derived from the capsule. However, this can only be done in patients with a functional spleen and normal complement system. The major advantage of the pneumococcal vaccine is the broad range of serotypes included in the vaccine. The major disadvantages are the unknown nature of the induced memory. The recently developed PCV overcomes the splenic dependency and shows "normal" immune response characteristic but has as major drawback the limited number of serotypes conjugated and the higher costs.

Children below the age of 2 years have an immature spleen and are vulnerable of infections with *Streptococcus pneumoniae*. The marginal zone B cells of children below the age of 2 years lack CD21 expression. This most likely explains the poor response of infant to PPS. Vaccination of children below the age of 2 years with the PPS vaccine is not warranted because of the immature spleen. In this group, vaccination with the PCV is highly recommended and effective.

In the past, infections with especially encapsulated bacteria caused problems in immunocompromised patients. Especially Hodgkin's disease patients were at high risk for severe infections, mostly due to splenectomy in combination with severe chemotherapy. Nowadays, because of better attention for infections, life-threatening infections are much less a problem during chemotherapy. In addition, splenectomy because of staging laparotomy in case of Hodgkin's disease is much less common. Still, infections with especially encapsulated bacteria can be responsible for the death of a patient receiving chemotherapy, not only during chemotherapy but also until approximately 3 years after chemotherapy [73].

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