HUMAN VACCINES & IMMUNOTHERAPEUTICS
2019, VOL. 15, NO. 2, 339–348
https://doi.org/10.1080/21645515.2018.1537744

RESEARCH PAPER

OPEN ACCESS

Effectiveness of *Haemophilus influenzae* type b vaccination after splenectomy - impact on selected immunological parameters

Ewelina Grywalska [a], Dorota Siwicka-Gieroba [b], Michał Mielnik [a], Martyna Podgajna [a], Krzysztof Gosik [a], Wojciech Dąbrowski [b], and Jacek Roliński [a]

[a]Department of Clinical Immunology and Immunotherapy, Medical University of Lublin, Lublin, Poland; [b]Department of Anesthesiology and Intensive Care, Medical University of Lublin, Lublin, Poland

ABSTRACT

Splenectomy is a surgery indicated in case of splenic rupture after injury, when there are tumors in the spleen, or as a treatment for certain diseases, such as idiopathic thrombocytopenic purpura and spherocytosis. The aims of the study were to assess the immunological response to the *Haemophilus influenzae* type b (Hib) vaccine and the post-vaccination changes in lymphocyte subsets and cell activation markers in splenectomized patients and healthy volunteers. Blood samples were collected from 25 patients that had undergone splenectomy and from 15 healthy, non-splenectomized volunteers. All participants received a single dose of Hib vaccine. The concentration of specific Hib antibodies was assessed by an enzyme-linked immunosorbent assay. Selected immune cell populations were evaluated using flow cytometry. The analysis of the antibody titers against Hib showed statistically significant differences in both groups. There was a significantly higher percentage ($p = 0.0012$) and absolute value ($p = 0.0003$) of natural killer T (NKT)-like cells (CD3+/CD16+ CD56+) in the study group, compared to the control group. The levels of natural killer (NK) and NKT cells did not change relative to the cause and age of splenectomy. The quantity and percentage of regulatory T (Treg) cells were higher in the study group compared to the control group ($p < 0.0001$). No significant correlations were found between the time elapsed since splenectomy, the age of the patients, and the Treg levels. Our study showed that spleen resection results in an important deterioration of Treg cells and Th17 cell balance which may contribute to an incomplete immunological response.

ARTICLE HISTORY
Received 29 July 2018
Revised 18 September 2018
Accepted 6 October 2018

KEYWORDS
Haemophilus influenzae; vaccine; splenectomy; immunology; immunological response; lymphocytes; treg cells; NKT-like cells

Introduction

The spleen is the largest lymphoid organ in the human body and plays an important role in the immunological response. The effects of splenectomy on the immune competence of an individual are not completely understood. Many studies have confirmed the association between asplenia or impaired splenic functions and morbidity and mortality. The lack of a correctly functioning spleen impairs mechanisms that are important for bacterial clearance and increases the risk of frequent infections. Splenectomised patients have a higher susceptibility to infections by encapsulated bacteria because of the absence of the phagocytic function of the spleen and the long-term impairment of the humoral response. Overwhelming post-splenectomy infection (OPSI) is triggered mainly by *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* type b (Hib) in splenectomised and hyposplenic individuals.[1] This severe infection occurs at an estimated incidence of 0.23–0.42% per year, with a mortality rate of 38–69%. Splenectomy correlates with an increased risk of lethal sepsis with multiple organ failure.[2] Long-term observations of asplenic patients indicate the occurrence of systemic infections and high mortality even 25 or more years after spleen resection, with the highest risk occurring during the first three years.

H.influenzae may be contracted at any age and is responsible for recurrent colonization of the human respiratory system. Chronic bacterial colonization may contribute to the progression of chronic pulmonary diseases and is associated with airflow obstruction and exacerbations of the disease.[3] The acquisition of new *H. influenzae*, *Moraxella catarrhalis*, *S. pneumoniae* or *Pseudomonas aeruginosa* strains has been linked to acute exacerbations in 20–30% of patients with chronic obstructive pulmonary disease (COPD).[4] The incidence of Hib diseases has been drastically lowered in all countries that have included Hib vaccines in their public immunization schedules.[5]

Hib vaccines stimulate the production of antibodies against Hib capsular polysaccharide antigens, which provoke the complement-dependent killing and phagocytosis of the bacteria.[6] In unvaccinated patients, protection against Hib is mediated by natural antibodies.[7] Production of natural antibodies is stimulated by Hib carriage and exposure to cross-reactive bacteria. To date, the effectiveness and safety of the Hib vaccine in patients with autoimmune diseases or after splenectomy has not been clearly established.[8]

CD4(+)T cells are essential for the immunological response and also take part in the pathogenesis of inflammatory diseases. In recent times, increasing attention has been paid to regulatory T cells (Tregs) and their ability to monitor the immune response

CONTACT Ewelina Grywalska ewelina.grywalska@gmail.com Department of Clinical Immunology and Immunotherapy, Medical University of Lublin, Poland
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/khvi.

© 2018 The Author(s). Published with license by Taylor & Francis Group, LLC
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

to self-antigens. Tregs are important in the suppression of excessive immune responses and distractions in development. They also play a key role in the pathogenesis of autoimmune and infectious diseases in humans and animals. Proliferation of natural Treg cells may be triggered by antigen stimulation, and their suppressive function is retained for a certain period of time. Natural killer T (NKT)-like cells are also significant immunological regulators against infectious diseases. NKT cells control bacterial clearance involving both innate and adaptive immunity. Persistent viral infections constantly stimulate the immune system and may result in the continuous activation of CD8 + T cells and subsequent immunopathology. The permanent contact with antigens can trigger a weaker functional response in exhausted T cells.

The aims of the study were to assess and the immunological response to the Hib vaccine and the post-vaccination changes in lymphocyte subsets and cell activation markers in splenectomized patients and healthy volunteers

Results

Table 1 (a) presents the characteristics of the study and control groups. Table 1 (b) presents significant differences (marked with *) in the percentage and concentration of lymphocyte subsets and serum anti-Hib antibody before and after Hib vaccination in the study and control groups.

Table 2 shows the change in the number of Th17 lymphocytes after vaccination in the study group (a) and the control group (b). The change was significant only in the study group ($p = 0.004$).

Figure 1 (a–l) presents graphs that illustrate the results obtained in the study group vs the control group before vaccination.

Figure 2 (a–d) presents graphs that illustrate the results obtained in the study group vs the control group after vaccination.

Adequate response to vaccination was defined as a minimum two-fold increase in specific anti-Hib antibody titers compared to pre-vaccination baseline titers.

This vaccination response criterion was met by 100% of patients (n = 25) and 100% of participants (n = 15) from the control group.

Before vaccination, there were no significant differences in the specific anti-Hib antibody titers between the study group and the control group ($p = 0.654879$; Figure 1 (a)) The specific anti-Hib antibody titers following vaccination were significantly higher in the control group compared to the study group ($p = 0.003123$; Figure 2 (a)).

Table 1. **(a)** Characteristics of the study and control groups. **(b)** Percentages of lymphocyte subsets and serum anti-*Haemophilus influenzae* type B (Hib) antibody concentration, and immunoglobulin levels before and after Hib vaccination in the study and control groups.

Parameter		Splenectomized patients (n = 25)	Control group (n = 15)
(a)			
Age	Mean ± SD	43.84 ± 16.47	44.33 ± 13.97
	Median (min-max)	42 (22–69)	50.00 (20–60)
Time since splenectomy (years)	Mean ± SD	9.12 ± 4.39	Not applicable
	Median (min-max)	9 (2–18)	
Age at splenectomy (years)	Mean ± SD	34.72 ± 17.19	Not applicable
	Median (min-max)	30 (10–65)	
Frequency of infections after splenectomy	Increased	17 (68%)	Not applicable
	Not increased	8 (32%)	

Parameter	N	Median	Lower quartile	Upper quartile	p
(b)					
Splenectomized patients					
Treg cells [%]					
before vaccination	25	11.54	9.99	12.23	0.000012*
after vaccination	25	8.72	7.92	9.10	
Th17 cells [%]					
before vaccination	25	0.85	0.54	1.14	0.000364*
after vaccination	25	1.96	1.40	3.20	
NK cells (CD3-/CD16+ CD56+) [%]					
before vaccination	25	12.21	8.19	16.73	0.007424*
after vaccination	25	17.66	13.42	20.51	
NKT-like cells (CD3+/CD16+ CD56+) [%]					
before vaccination	25	4.66	2.92	6.10	0.032428*
after vaccination	25	2.42	1.99	4.50	
anti-Hib antibodies [ug/ml]					
before vaccination	25	0.67	0.53	1.46	< 0.000001*
after vaccination	25	4.10	2.14	5.40	
Control group					
Treg cells [%]					
before vaccination	15	5.50	4.20	6.93	0.093840
after vaccination	15	4.49	4.00	5.71	
Th17 cells [%]					
before vaccination	15	0.92	0.79	1.89	0.030909*
after vaccination	15	1.80	1.53	2.88	
NK cells (CD3-/CD16+ CD56+) [%]					
before vaccination	15	24.68	11.82	28.68	0.078293
after vaccination	15	17.14	13.62	18.99	
NKT-like cells (CD3+/CD16+ CD56+) [%]					
before vaccination	15	2.71	1.08	7.30	0.191447
after vaccination	15	2.09	1.06	3.16	
anti-Hib antibodies [ug/ml]					
before vaccination	15	0.75	0.29	4.58	< 0.000001*
after vaccination	15	7.35	7.23	7.35	

Table 2. Change in Th17 cell number before and after vaccination in the study group (a) and the control group (b). * Indicates statistical significance.

	Median	Lower quartile	Upper quartile	p
(a) Th17 cell number in the study group (n = 25)				
Before vaccination	0.021	0.013	0.045	0.004*
After vaccination	0.051	0.030	0.077	
(b) Th17 cell number in the control group (n = 15)				
Before vaccination	0.023	0.016	0.043	0.061
After vaccination	0.060	0.030	0.075	

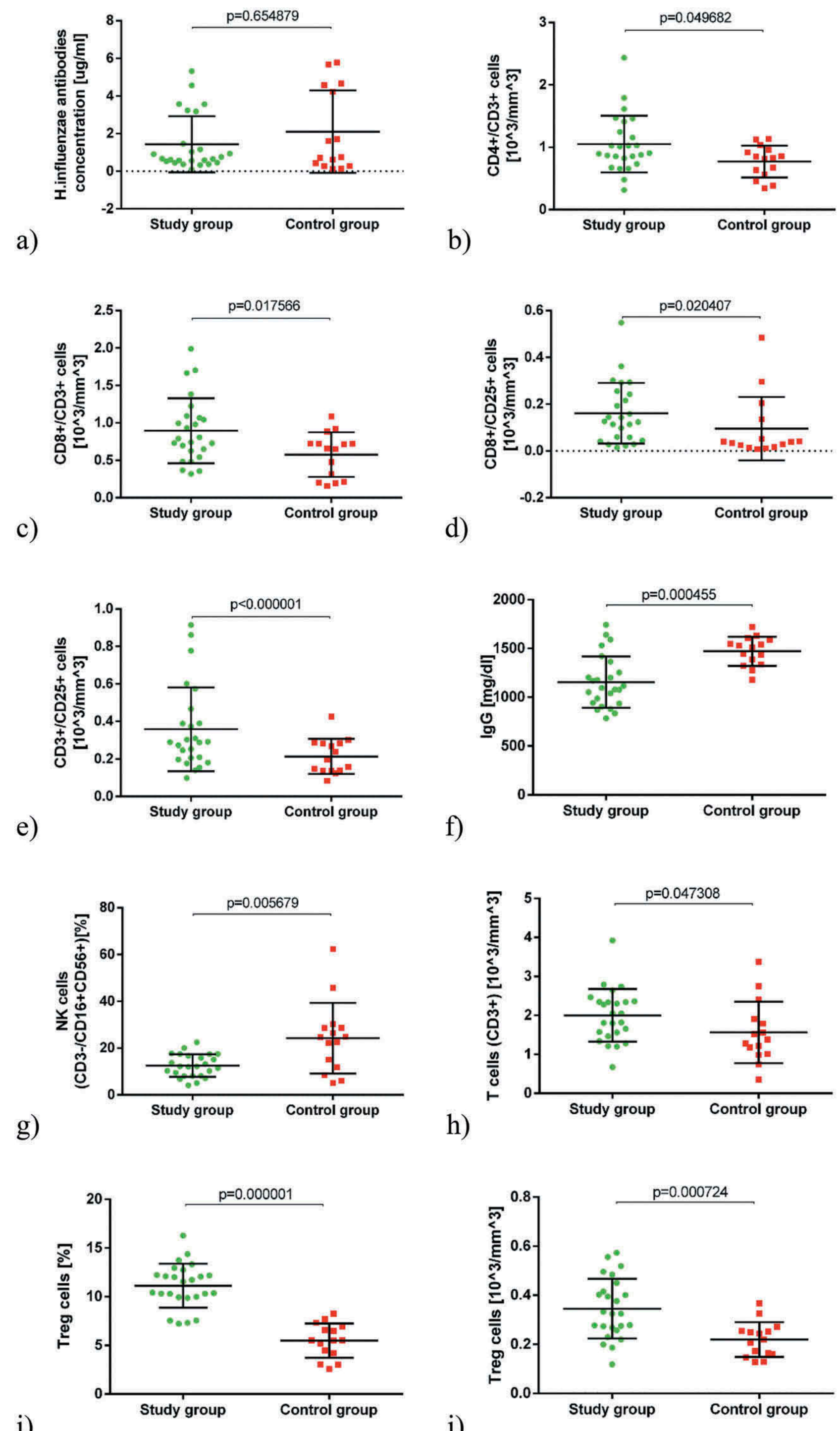

Figure 1. Comparison of selected parameters in the study group vs the control group before vaccination: (a) Specific anti H.influenzae antibody titers (p = 0.654879); (b) Absolute counts of CD4+ CD3+ cells (p = 0.049682); (c) Absolute counts of CD8+ CD3+ cells (p = 0.017566); (d) Absolute counts of CD8+ CD25+ cells (p = 0.020407); (e) Absolut counts of CD3+ CD25+ cells (p < 0.000001); (f) Absolut counts of IgG antibodies (p = 0.000455); (g) Percentage of NK cells (CD3-/CD16 + CD56+) (p = 0.005679); (h) Absolute counts of T cells (CD3+) (p = 0.047308); (i) Percentage of treg cells (p = 0.000001); (j) Absolute counts of Treg cells (p = 0.000724).

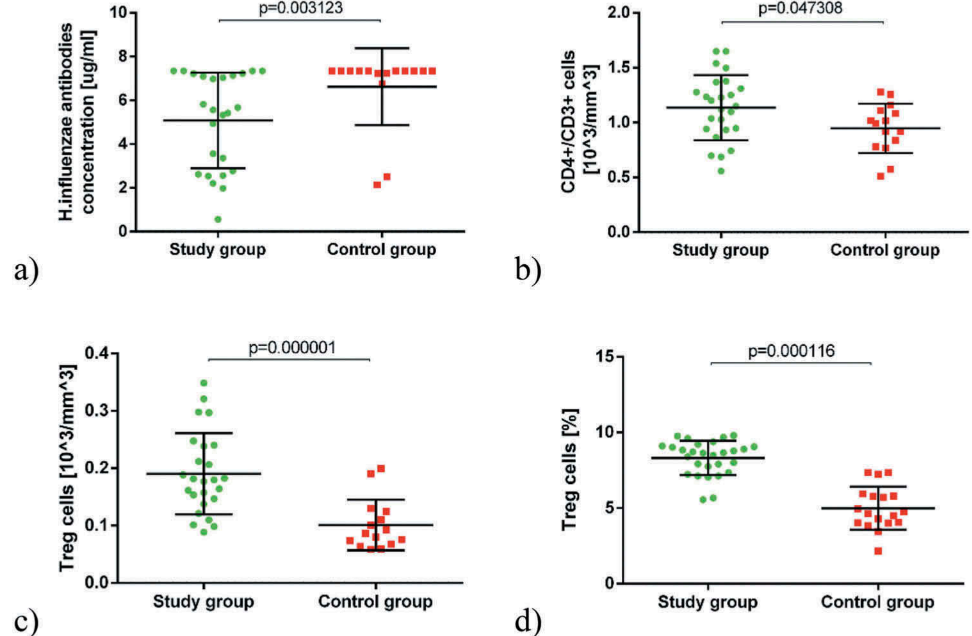

Figure 2. Comparison of the selected parameters in the study group vs the control group after vaccination: (a) Specific anti Hib antibody titers ($p = 0.003123$); (b) Absolute counts of CD4+ CD3+ cells ($p = 0.047308$); (c) Absolute counts of Treg cells ($p = 0.000001$); (d) Percentage of Treg cells ($p = 0.000116$).

We also measured the specific anti-Hib antibody titers 12–14 months after vaccination. We observed a statistically significantly lower antibody titer after 12–14 months than after 4–6 weeks in splenectomized patients ($p = 0.0001$; Figure 3 (a)). There was no such difference in participants from the control group ($p = 0.0625$; Figure 3 (b)). The vaccination response was sustained by 19 out of 25 patients (76%) and by the 15 (100%) participants from the control group.

Table 3 shows the results of the correlation analysis between the number of Treg cells and the anti-Hib antibody titers at different time points for the study and the control groups. Significant correlations were found in the study group between the anti-Hib antibody titers 12–14 months after vaccination and the percentage of Treg cells before vaccination ($R = 0.397$; $p = 0.049$), and the number of Treg cells after vaccination ($R = 0.502$; $p = 0.011$). In the control group, the only significant correlation was between the anti-Hib antibody titers and the number of Treg cells before vaccination ($R = -0.539$; $p = 0.038$).

Significant correlations were found in the study group, between the percentage of NKT-like cells (CD3+/CD16 + CD56+) after vaccination and the absolute counts of anti-Hib antibody titers (μg/ml) before vaccination ($R = 0.490769$; $p = 0.012740$; Figure 4 (a)), 4–6 weeks after vaccination ($R = 0.408864$; $p = 0.042423$; Figure 4 (c)), and 12–14 months after vaccination ($R = 0.557692$; $p = 0.003773$; Figure 4 (e)).

a. the absolute counts of anti-Hib antibody titers before vaccination [μg/ml] and the percentage of NKT-like cells (CD3+/CD16+ CD56+) [%] after vaccination ($R = 0.490769$; $p = 0.012740$; Figure4 (a));
b. the absolute counts of anti-Hib antibody titers 4–6 weeks after vaccination [μg/ml] and the percentage

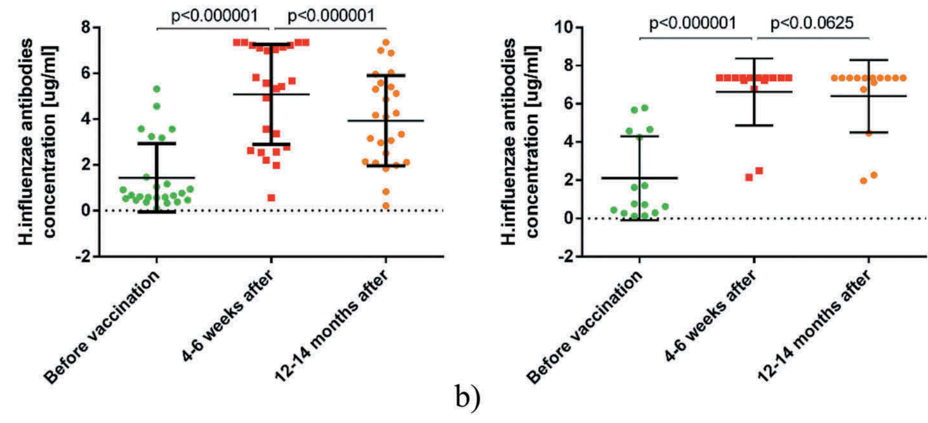

Figure 3. Comparison of specific anti Hib antibody titers at 3 timepoints during the study in the (a) study group ($p < 0.000001$; $p < 0.000001$, respectively) and (b) control group ($p < 0.000001$; $p = 0.0625$, respectively).

Table 3. Correlations (Spearman rho) between the percentage (%) and number (#) of Treg cells, and anti-HiB antibody titers (µg/ml) before vaccination, 4–6 weeks after vaccination, and 12–14 months after vaccination in the study (a) and control (b) groups. * Indicates statistical significance.

		Anti-Hib before	Anti-Hib 4–6 weeks	Anti-Hib 12–14 months
(a) Study group (n = 25)				
Tregs before (%)	Rho p (2-tailed)	0.122 0.561	0.158 0.452	0.397 0.049*
Tregs after (%)	Rho p (2-tailed)	0.093 0.659	0.179 0.392	0.395 0.050
Tregs before (#)	Rho p (2-tailed)	0.033 0.875	0.034 0.874	0.192 0.359
Tregs after (#)	Rho p (2-tailed)	0.173 0.408	0.242 0.245	0.502 0.011*
(b) Control group (n = 15)				
Tregs before (%)	Rho p (2-tailed)	0.479 0.071	0.336 0.221	0.336 0.221
Tregs after (%)	Rho p (2-tailed)	−0.093 0.742	0.374 0.169	0.374 0.169
Tregs before (#)	Rho p (2-tailed)	−0.539 0.038*	−0.132 0.640	−0.132 0.640
Tregs after (#)	Rho p (2-tailed)	0.229 0.413	−0.026 0.928	−0.026 0.928

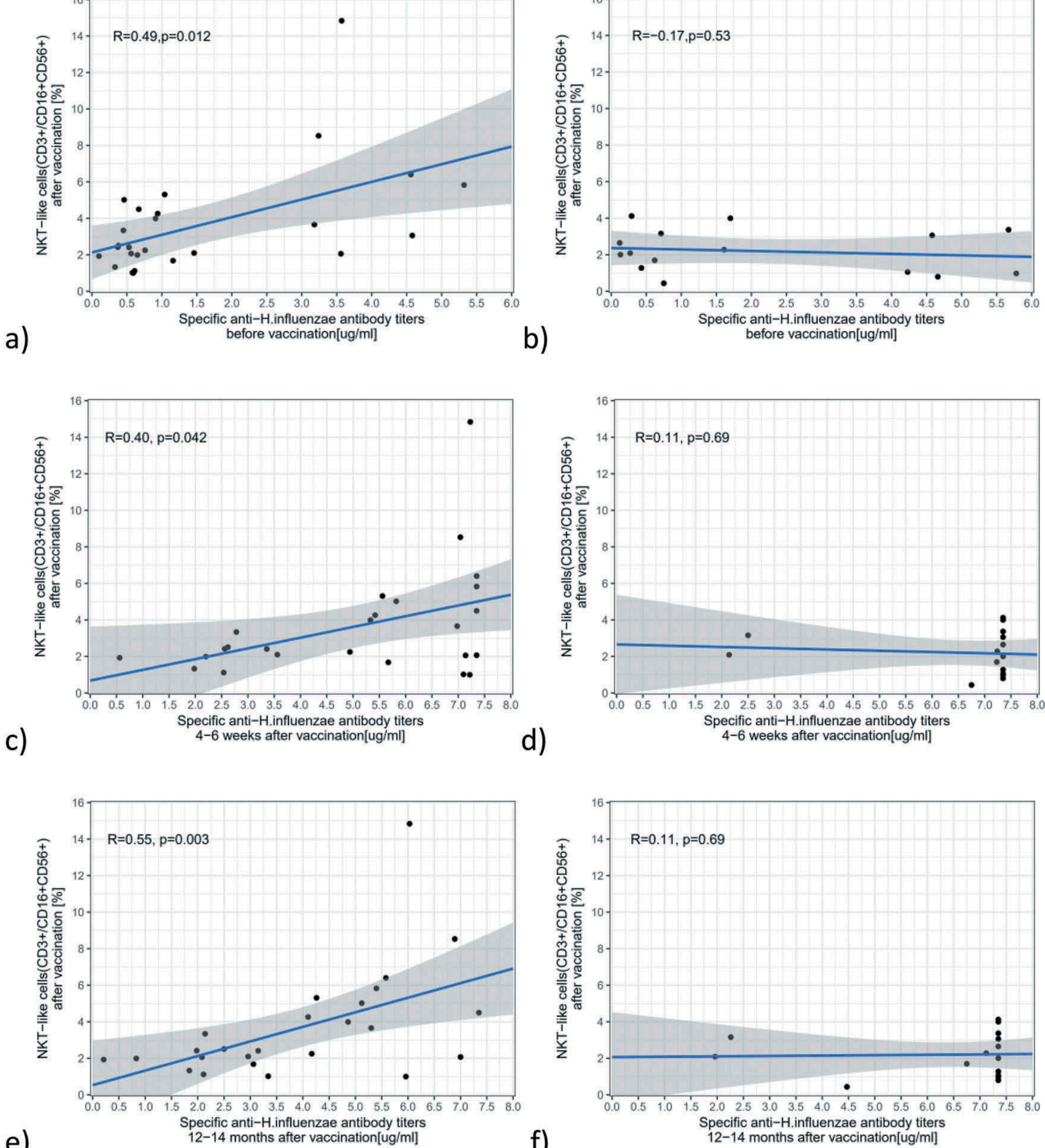

Figure 4. Correlation between the absolute counts of anti-Hib antibody titers before vaccination (µg/ml) and the percentage of NKT-like cells (CD3+/CD16+ CD56+) after vaccination in the study group (a; R = 0.49; p = 0.012) and control group (b; R = 0.17; p = 0.53); Correlation between the absolute counts of anti-Hib antibody titers 4–6 weeks after vaccination [µg/ml] & NKT-like cells (CD3+/CD16+ CD56+) [%] after vaccination in the study group (c; R = 0.40, p = 0.042) and control group (d; R = 0.11; p = 0.69); Correlation between the absolute counts of anti-Hib antibody titers 12–14 months after vaccination [µg/ml] & NKT-like cells (CD3+/CD16+ CD56 +) [%] after vaccination in the study group (e; R = 0.55, p = 0.003) and control group (f; R = 0.11; p = 0.69).

of NKT-like cells (CD3+/CD16+ CD56+) [%] after vaccination (R = 0.408864; p = 0.042423; Figure 4 (c));
c. the absolute counts of anti-Hib antibody titers 12–14 months after vaccination [µg/ml] and the percentage of NKT-like cells (CD3+/CD16+ CD56+) [%] after vaccination (R = 0.557692; p = 0.003773; Figure 4 (e)).

A significant positive correlation was also found between the number of NKT cells after vaccination and the anti-Hib antibody titers (µg/ml) 12–14 months after vaccination in the study group (R = 0.605; p = 0.001) but not in the control group (R = −0.009; p = 0.976; Table 4).

Discussion

In this study, we assessed the immunologic response against the Hib vaccine in splenectomized patients compared to healthy controls. Splenectomized patients are at a higher risk of infection by encapsulated bacteria compared to the general population. However, the way in which the lack of a spleen affects the immune response remains unclear. While some studies have reported an inadequate production of antibodies in splenectomized patients after immunization with polysaccharide vaccines,[9] others have found a deficiency only in some classes of immunoglobulins.[10,11] In our study, both splenectomized and control participants showed an adequate immune response after vaccination, but antibody titers decreased in the splenectomized patients after 12–14 months.

One explanation for the impaired immune system of splenectomized patients is a loss of memory B cells and IgM antibodies. Memory B cells are known to produce antibodies against polysaccharide antigens, and some studies have shown that removing the spleen results in a loss of these cells.[12] Wasserstorm et al. found that the IgG antibody titers were not different in splenectomized patients compared to healthy controls after vaccination with a pneumococcal vaccine. However, the concentration of IgM antibodies and the number of memory B cells differed in patients according to the reason for splenectomy. Only splenectomized patients with autoimmune disease had IgM titers and memory B cell number significantly lower compared to healthy controls.[10] Mikoluc et al. measured the concentration of specific Hib antibodies in asplenic children and young adults before and after immunization with a Hib conjugate vaccine. Vaccination resulted in an increase in antibody titers, albeit moderate. Interestingly, the immunization seemed less effective in children who had their spleen removed after the age of six.[13]

Results such as these suggest that the reason for the immunologic differences between the patients and controls is complex and involves factors surrounding the splenectomy surgery. In particular, it seems that patients splenectomized later in life show a diminished effectiveness to vaccination and greater alterations in their immune response. Some authors have suggested that there is likely a loss of sites outside the spleen that are necessary for the maintenance of memory B cells and that happens with age.[10] In our study, all the splenectomized patients had their spleens removed after the age of 6, which could be a reason for their short-lived antibody response.

Although the antibody concentration against the PRP antigen has been observed to decrease over time, it is not known whether this makes the patients more susceptible to infection.[13-15] In his observations on patients with thalassemia with and without a spleen, Cimaz et al. found an increase in antibody concentration 2 months after vaccination, followed by a gradual decrease over time. There were no differences between splenectomized patients and those with a spleen, either in the postvaccination antibody increase or in antibody persistence.[15] In our study, the antibodies of the study group decreased after 12–14 months. A decrease in antibody concentration after conjugate vaccines does not necessarily mean an increase in susceptibility to infection because these vaccines elicit immunologic memory.[16] However, it is possible that patients without a spleen may need higher concentrations of antibodies to avoid infection.[15,17]

Current guidelines recommend one dose of conjugate Hib vaccine for splenectomized patients, including patients who were previously vaccinated, but booster shots are not recommended.[14] The risk of OPSI is highest immediately after surgery and decreases with time. Thirty percent of OPSI cases happen during the first year, and 50% within the first 2 years.[18,19] In our study, 34% of patients had lower anti-Hib antibodies already after the first year. Some researchers have suggested giving patients a booster shot ever 5 years to maintain long-term protection.[15]

At all three time points during our study, the percentage of NKT cells after vaccination had a significant, positive correlation with the antibody titers in the patients. There was also a significant, positive correlation between the number of NKT cells after vaccination and the antibody titers at 12–14 months after vaccination. These correlations were not statistically significant in the control group, suggesting a possible role of NKT cells in splenectomized patients. Furthermore, although an adequate vaccination response was reached by 100% of participants in both groups, only 76% of splenectomized patients sustained the initial response after one year. This suggests a strong but short-lived antibody production in the patients.

The action of NKT-like cells is immediate and contributes to the stimulation of other cells of the immune system, such as NK cells, conventional CD4+ or CD8 + T cells, dendritic cells, and B lymphocytes.[20]

NKT cells are characterized by the instantaneous and rapid secretion of cytokines, including interferon (IFN)-gamma, IL-4,

Table 4. Correlations (Spearman rho) between anti-Hib antibody titers (µg/ml) 12–14 months after vaccination and the percentage (%) and number (#) of NKT cells before and after vaccination in the study and control groups. * Indicates statistical significance.

		NKT cells (%)		NKT cell number (#)	
		Before vaccination	After vaccination	Before vaccination	After vaccination
Study group (n = 25)	Rho p (2-tailed)	0.132 0.528	0.558 0.004*	0.215 0.301	0.605 0.001*
Control group (n = 15)	Rho p (2-tailed)	−0.071 0.801	0.111 0.695	−0.223 0.424	−0.009 0.976

−2, −5, −6, −10, −13, tumor necrosis factor (TNF), transforming growth factor (TGF)-beta, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[21-23]

In addition, recent studies have shown that the population of type-I NKT cells (also known as invariant NKT, or iNKT, cells) with the phenotype CD4-NK1.1 can rapidly secrete a significant amount of IL-17.[24-27] This rapid cytokine release is possible, at least in part, because of pre-formed cytokine-encoding mRNA, such as for IFN-gamma and IL-4. In this way, these cells can start cytokine secretion faster, without the need for gene transcription.[28]

NKT cells are an important part of the acquired immune system. They fill the gap in the repertoire of antigens recognized by conventional T cells, commonly recognizing peptide and non-lipid antigens. The ability of NKT cells to recognize bacterial lipids indicates the role of these cells in antibacterial resistance. Thus, NKT cells can serve as regulatory cells and, potentially, effector cells in various types of immune response, including those directed against infectious agents.[20,29-31]

A subset of iNKT cells have been observed to behave like follicular T helper cells and have thus been called follicular NKT helper cells (NKT_{FH}). Certain studies have shown that under some circumstances, iNKT cells can directly stimulate B cells and induce B cell proliferation and antibody production in high titers.[32-34]

Barral et al. showed that when stimulated with α-galactosylceramide (αGalCer), a known iNKT antigen, iNKT cells can induce B cell proliferation in vivo. These B cells produced a high concentration of IgM antibodies and early-switched antibodies and developed in extrafollicular foci.[32] Later, Tonti et al. immunized mice lacking classic CD4 + T helper cells with protein antigens mixed with αGalCer. They observed that the stimulated iNKT cells provided direct help to B cells and stimulated antibody production. However, this response was short-lived. King et al. observed similar results in mice and described this as a hybrid between T-dependent and T-independent B cell activation mechanisms. iNKT cells provided direct help to B cells and stimulated a robust antibody production, but without memory formation.

Most of these observations have been made in controlled laboratory conditions, rather than in the real-life scenario of immune response to vaccination like the one presented in this work. In our study, the number of NKT cells was positively correlated to the antibody titer in the splenectomized patients, and antibody titers decreased over time in this population. Thus, one possible explanation for the difference with the healthy participants could be the effect of NKT_{FH} cells acting in splenectomized patients.

H. influenzae is a Gram-negative airborne rod, mainly associated with respiratory infections and meningitis, especially in people who are immunosuppressed.[6]

The main virulence factors of *H. influenzae* include a capsular polysaccharide, which has antiphagocytic properties and is the most important virulence factor of subtype b; membrane lipo-oligosaccharides, which are involved in bacterial adherence, invasiveness, and damage to ciliated epithelium; and immunoglobulin A (IgA) protease, which neutralizes antibodies against bacteria.[35,36]

NKT cells recognize glycolipid antigens, such as glycosylceramides, present in the cell wall of Gram-negative bacteria.[37-39]

NKT cells can recognize lipid antigens and thus broaden the repertoire of antigens recognized by conventional T cells. In addition, in spite of the limited diversity of T-cell receptors (TCR) in NKT cells, they recognize many different microorganism antigens, which points to the high plasticity of their TCRs.[20]

The results we obtained show that the percentage of NKT cells is closely correlated to the concentration of specific antibodies against Hib in splenectomized patients. It can be concluded that in the absence of the spleen, the number of NKT cells increases after administration of the vaccine to fill the gap in response to Hib.

There are a few limitations associated with our study. First, we did not investigate variations in B cell populations, which could give further insight into the differences between the study and control groups. Antibody response is only one part of the immunologic reaction to vaccines, and low antibody concentration does not necessarily mean a loss of protection because immune memory can persist. Second, our study measured antibody titers up to 12–14 months after vaccination, which revealed a reduction in antibody titers in the study group only. However, other studies have shown that healthy individuals also experience a decrease in antibody titers over time. A longer follow-up time might have revealed this reduction in the control group as well. Third, our study included only patients splenectomized later in life because of trauma. Yet, other studies have shown that the reason for splenectomy and the age of the patients at the time of surgery may have an impact on their immunologic response against vaccines. Therefore, the results observed in our study might not be applicable to all subsets of splenectomized patients.

Materials and methods

Blood samples were collected for analysis from splenectomised patients (study group) and healthy volunteers (control group). The study group consisted of 25 patients aged 19–72 years (mean age 44.92 years), who had undergone urgent splenectomy caused by abdominal trauma between 2013–2016 and were diagnosed and treated at Clinical Hospital No. 4 in Lublin. Upper respiratory tract infections (URTI) post-splenectomy, occurring with a frequency of 4 or more infections per year treated with antibiotics, were reported in 86.7 % of patients. The control group consisted of 15 volunteers with a healthy spleen and aged 20–67 years (mean age 45.73 years).

All the study participants were immunized with a single dose of Hib vaccine according to standard protocol. The participants from the control group were immunized at their own request – each of them had a family member with immune deficiencies caused by oncological treatment and wanted to prevent them from infection. Neither the splenectomy patients nor the healthy volunteers received any immunomodulating agents or hormonal preparations. They did not show any signs of infection for at least 3 months before the study, did not undergo blood transfusion, and did not present with autoimmune conditions or allergies.

This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of

Helsinki) for experiments involving humans and approved by the Ethics Committee of the Medical University of Lublin (decision no. KE-0254/290/2014).

Materials

All study participants received the Hib conjugate vaccine Hiberix (GlaxoSmithKline). Every dose was composed of 10 µg of purified Hib capsular polysaccharide covalently bound to 25 µg of tetanus toxoid. The vaccine was supplied as a lyophilized powder and was reconstituted before use according to the manufacturer's instructions.

Peripheral blood (PB) was drawn from the basilic vein of all study participants before and 4–6 weeks after vaccination. To assess serum-specific Hib antibody titers, 3 ml of blood were collected using blood-collection tubes coated with a clotting activator. To evaluate the frequencies of selected lymphocyte subsets, 5 ml of PB were collected and mixed with the anticoagulant EDTA. Serum samples were stored at −70°C until the analysis of Hib antibody titer was carried out.

Assessment of serum anti-hib antibody concentration

The assessment of serum anti-Hib antibody titer was performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Demeditec), according to manufacturer's instructions. An automatic VICTOR3 reader (Perkin Elmer, USA) was used for the interpretation of the results.

Assessment of basic lymphocyte subsets, NK, and nkt-like cells

Samples were prepared for cytometric analysis from freshly collected PB. The samples were incubated with the following set of monoclonal antibodies: anti-CD3 FITC, anti-CD3 PECy5, anti-CD4 FITC, anti-CD8 FITC, anti-CD8 PE, anti-CD19 FITC, anti-CD19 PE, anti-CD25 PECy5, CD45RA PE, CD45RO PE, and anti-CD3 FITC/anti-CD16 PE/anti-CD56 PE (BD Pharmingen, USA). Erythrocytes were removed from the samples by adding a lysing solution (FACS Lysing Solution, Becton Dickinson, USA). The immunophenotype of the PB cells was determined using a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with a 488-nm argon laser. The results were analyzed with CellQuest Pro software (Becton Dickinson, USA).[26,31]

Assessment of regulatory t cells

Treg CD4(+)CD25(+)Foxp3(+) cells were prepared for visualization from the fresh blood samples at the time of sample collection. The cells were stained according to the manufacturer's instructions. After the standard incubation with antibodies against surface markers, the cells were fixed and permeabilized using the FoxP3 Fix/Perm Buffer and FoxP3 Perm Buffer (BioLegend, San Diego, CA, USA). Next, the cells were incubated with antibodies directed against the intracellular protein FoxP3, using the anti-Human FoxP3 (Pacific Blue) monoclonal antibody (BioLegend, San Diego, CA, USA). Immediately after incubation, the cells were analyzed using a Becton Dickinson Canto II flow cytometer (Becton Dickinson, San Diego, CA, USA) and FACSDiva™ Software (Becton Dickinson, San Diego, CA, USA).

Assessment of th17 lymphocytes

PB mononuclear cells (PBMCs) were aseptically isolated from the blood samples using standard density gradient centrifugation (Gradisol L; Aqua Medic, Lodz, Poland). For the detection of Th17 cells, PBMCs were resuspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 U/ml of penicillin (Sigma-Aldrich, St. Louis, MO, US), and 100 µg/ml of streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cells were stimulated with 25 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma Chemical C., St. Louis, MO, USA) and 1 µg/ml of ionomycin (Sigma Aldrich, St. Louis, MO, USA) at 37°C in a 5% CO_2 atmosphere for 5 h. The cells were stimulated in the presence of 10 µg/ml of brefeldin A (Sigma-Aldrich, St. Louis, MO, USA), which inhibits the intracellular transport processes resulting in the accumulation of cytokine proteins in the Golgi complex. Next, PBMCs were isolated, washed with PBS solution, and diluted to a final concentration of 1×10^6 cells/ml. The number of viable leukocytes was determined using a 1% trypan blue exclusion solution. Later, the mononuclear cells were stained with anti-CD3 CyChrome- and anti-CD4 FITC- conjugated monoclonal antibodies (Becton Dickinson, San Diego, CA, USA). The cell membranes were permeabilized using a Cytofix/Cytoperm Kit (BD Pharmingen, San Jose, CA, USA) for 15 min at 4°C. Subsequently, the cells were washed twice with PBS. The permeabilized cells were stained with a PE-conjugated anti-human interleukin (IL)-17A monoclonal antibody (eBioscience, San Diego, CA, USA). The cells were then washed twice with PBS and immediately analyzed with a Becton Dickinson Canto II flow cytometer (Becton Dickinson, San Diego, CA, USA) and FACSDiva™ Software (Becton Dickinson, San Diego, CA, USA). The results are presented as the percentage of CD45+ cells stained with the antibodies. The percentage of positive cells was calculated by comparing with the control group. Background fluorescence was determined using isotype-matched, directly conjugated mouse anti IgG1/IgG2α monoclonal antibodies. The samples were gated on the forward scatter versus the side scatter to exclude debris and cell aggregates.[40]

Statistics

All analyses were performed using Statistica 10.0 (StatSoft Inc., 2011). The distribution of variables was evaluated with the Shapiro-Wilk test. n case of unconfirmed normal distribution, the nonparametric Mann-Whitney U test or the Kruskal-Wallis rank test was used. The Wilcoxon test was used to assess antibody parameters. Statistical significance was considered at $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

Funding

This work was supported by research grants no. UMO-2016/21/B/NZ6/02279 and no. UMO-2012/05/B/NZ6/00792 of the Polish National Science Centre, and grants no. MN534 and no. DS460 of the Medical University of Lublin. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript; Narodowe Centrum Nauki [UMO-2016/21/B/NZ6/02279 and UMO-2012/05/B/NZ6/00792]; Uniwersytet Medyczny w Lublinie [MN534 and DS460]

ORCID

Ewelina Grywalska http://orcid.org/0000-0002-0451-4741
Michał Mielnik http://orcid.org/0000-0003-4473-5319
Martyna Podgajna http://orcid.org/0000-0002-2863-5142
Jacek Roliński http://orcid.org/0000-0001-5596-2651

References

1. Di Sabatino A, Carsetti R, Corazza GR. Post-splenectomy and hyposplenic states. Lancet (London, England). 2011;378(9785):86–97. doi:10.1016/S0140-6736(10)61493-6.
2. Edgren G, Almqvist R, Hartman M, Utter GH. Splenectomy and the risk of sepsis: a population-based cohort study. Ann Surg. 2014;260(6):1081–1087. doi:10.1097/SLA.0000000000000439.
3. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. N Engl J Med. 2008;359(22):2355–2365. doi:10.1056/NEJMra0800353.
4. Sethi S, Evans N, Grant BJB, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med. 2002;347(7):465–471. doi:10.1056/NEJMoa012561.
5. Mandell G, Bennett J, Dolin R. Haemophilus infections. In: Principles and practice of infectious diseases. Philadelphia (PA): 6th Churchill Livingstone. http://www.us.elsevierhealth.com/product.jsp?isbn=9780443068393&elsca1=doodys&elsca2=PDF&elsca3=Mandell9780443068393&elsca4=frontmatter
6. Nix EB, Hawdon N, Gravelle S, Biman B, Brigden M, Malik S, McCready W, Ferroni G, Ulanova M. Risk of invasive haemophilus influenzae type b (Hib) disease in adults with secondary immunodeficiency in the post-Hib vaccine era. Clin Vaccine Immunol. 2012;19(5):766–771. doi:10.1128/CVI.05675-11.
7. Hawdon N, Nix EB, Tsang RSW, Ferroni G, McCready WG, Ulanova M. Immune response to haemophilus influenzae type b vaccination in patients with chronic renal failure. Clin Vaccine Immunol. 2012;19(6):967–969. doi:10.1128/CVI.00101-12.
8. Teo E, House H, Lockhart K, Purchuri SN, Pushparajah J, Cripps AW, van Driel ML. Haemophilus influenzae oral vaccination for preventing acute exacerbations of chronic bronchitis and chronic obstructive pulmonary disease. Cochrane Database Syst Rev. 2014;(9):CD010010. doi:10.1002/14651858.CD010010.pub2.
9. Konradsen HB, Rasmussen C, Ejstrud P, Hansen JB. Antibody levels against streptococcus pneumoniae and haemophilus influenzae type b in a population of splenectomized individuals with varying vaccination status. Epidemiol Infect. 1997;119(2):167–174. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2808838/.
10. Wasserstrom H, Bussel J, Lim -LC-L, Cunningham-Rundles C. Memory B cells and pneumococcal antibody after splenectomy. J Immunol. 2008;181(5):3684–3689. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919219/.
11. Molrine DC, Siber GR, Samra Y, Shevy DS, MacDonald K, Cieri R, Ambrosino DM. Normal IgG and impaired IgM responses to polysaccharide vaccines in asplenic patients. J Infect Dis. 1999;179(2):513–517. doi:10.1086/314582.
12. Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter -H-H, Berner R, Peters A, Boehm T, Plebani A, et al. Human immunoglobulin M memory B cells controlling streptococcus pneumoniae infections are generated in the spleen. J Exp Med. 2003;197(7):939–945. doi:10.1084/jem.20022020.
13. Mikoluc B, Motkowski R, Käyhty H, Heropolitanska-Pliszka E, Pietrucha B, Bernatowska E. Antibody response to haemophilus influenzae type-b conjugate vaccine in children and young adults with congenital asplenia or after undergoing splenectomy. Eur J Clin Microbiol Infect Dis. 2012;31(5):805–809. doi:10.1007/s10096-011-1378-8.
14. Bonanni P, Grazzini M, Niccolai G, Paolini D, Varone O, Bartoloni A, Bartalesi F, Santini MG, Baretti S, Bonito C, et al. Recommended vaccinations for asplenic and hyposplenic adult patients. Hum Vaccin Immunother. 2017;13(2):359–368. doi:10.1080/21645515.2017.1264797.
15. Cimaz R, Mensi C, D'Angelo E, Fantola E, Milone V, Biasio LR, Carnelli V, Zanetti AR. Safety and immunogenicity of a conjugate vaccine against haemophilus influenzae type b in splenectomized and nonsplenectomized patients with cooley anemia. J Infect Dis. 2001;183(12):1819–1821. doi:10.1086/320727.
16. Heath PT, Booy R, Azzopardi HJ, Slack MP, Bowen-Morris J, Griffiths H, Ramsay ME, Deeks JJ, Moxon ER. Antibody concentration and clinical protection after hib conjugate vaccination in the united kingdom. JAMA. 2000;284(18):2334–2340. doi:10.1001/jama.284.18.2334.
17. Rubin LG. Anticapsular antibody requirements for protection against experimental haemophilus influenzae type b bacteremia after splenectomy. Infect Immun. 1988;56(4):984–986. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC259402/.
18. Martino C, Gallone MS, Quarto M, Germinario C, Tafuri S. Immunization coverage among splenectomized patients: results of an ad hoc survey in puglia region (South of Italy). Hum Vaccin Immunother. 2016;12(5):1277–1279. doi:10.1080/21645515.2015.1138025.
19. Newland A, Provan D, Myint S. Preventing severe infection after splenectomy. BMJ. 2005;331(7514):417 LP–418. doi:10.1136/bmj.331.7514.417.
20. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the "Swiss-Army knife" of the immune system. Curr Opin Immunol. 2008;20(3):358–368. doi:10.1016/j.coi.2008.03.018.
21. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol. 2007;25:297–336. doi:10.1146/annurev.immunol.25.022106.141711.
22. Sakuishi K, Oki S, Araki M, Porcelli SA, Miyake S, Yamamura T. Invariant NKT cells biased for IL-5 production act as crucial regulators of inflammation. J Immunol. 2007;179(6):3452–3462.
23. Torina A, Guggino G, La Manna MP, Sireci G. The janus face of NKT cell function in autoimmunity and infectious diseases. Int J Mol Sci. 2018;19(2). doi:10.3390/ijms19020440.
24. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. Nat Immunol. 2010;11(3):197–206. doi:10.1038/ni.1841.
25. Michel M-L, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, Wong C-H, Schneider E, Dy M, Leite-de-Moraes MC. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. J Exp Med. 2007;204(5):995–1001. doi:10.1084/jem.20061551.
26. Klatka J, Grywalska E, Hymos A, Krasowska E, Mielnik M, Siwicka-Gieroba D, Markowicz J, Trojanowski P, Olszański W, Roliński J. Subpopulations of natural killer-T-like cells before and after surgical treatment of laryngeal cancer. Cent Eur J of Immunology. 2017;42(3):252–258. doi:10.5114/ceji.2017.70967.
27. Rachitskaya AV, Hansen AM, Horai R, Li Z, Villasmil R, Luger D, Nussenblatt RB, Caspi RR. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. J Immunol. 2008;180(8):5167–5171.
28. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang Z-E, Gapin L, Kronenberg M, Locksley RM. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med. 2003;198(7):1069–1076. doi:10.1084/jem.20030630.
29. Terabe M, Berzofsky JA. The role of NKT cells in tumor immunity. Adv Cancer Res. 2008;101:277–348. doi:10.1016/S0065-230X(08)00408-9.

30. Hodge G, Hodge S. Steroid resistant CD8(+)CD28(null) NKT-like pro-inflammatory cytotoxic cells in chronic obstructive pulmonary disease. Front Immunol. 2016;7:617. doi:10.3389/fimmu.2016.00617.
31. Nowicka D, Grywalska E, Fitas E, Mielnik M, Rolinski J. NK and NKT-like cells in patients with recurrent furunculosis. Arch Immunol Ther Exp (Warsz). 2017 December. doi:10.1007/s00005-017-0500-8.
32. Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, Besra GS, Cerundolo V, Batista FD. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. Proc Natl Acad Sci U S A. 2008;105(24):8345–8350. doi:10.1073/pnas.0802968105.
33. Tonti E, Fedeli M, Napolitano A, Iannacone M, von Andrian UH, Guidotti LG, Abrignani S, Casorati G, Dellabona P. Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. J Immunol. 2012;188(7):3217–3222. doi:10.4049/jimmunol.1103501.
34. King IL, Fortier A, Tighe M, Dibble J, Watts GF, Veerapen N, Haberman AM, Besra GS, Mohrs M, Brenner MB, Leadbetter EA. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an interleukin 21- dependent manner. Nat Immunol. 2011;13(1). doi:10.1038/ni.2172.
35. Akeda Y, Koizumi Y, Takanami Y, Sumino S, Hattori Y, Sugizaki K, Mitsuya N, Oishi K. Comparison of serum bactericidal and antibody titers induced by two haemophilus influenzae type b conjugate vaccines: A phase III randomized double-blind study. Vaccine. 2018;36(12):1528–1532. doi:10.1016/j.vaccine.2018.02.011.
36. Furuya Y, Kirimanjeswara GS, Roberts S, Racine R, Wilson-Welder J, Sanfilippo AM, Salmon SL, Metzger DW. Defective anti-polysaccharide IgG vaccine responses in IgA deficient mice. Vaccine. 2017;35(37):4997–5005. doi:10.1016/j.vaccine.2017.07.071.
37. Shimamura M, Yamamura M, Nabeshima T, Kitano N, van Den Elzen P, Yesilkaya H, Andrew P, Illarionov P. Activation of invariant natural killer T cells stimulated with microbial alpha-mannosyl glycolipids. Sci Rep. 2017;7(1):9703. doi:10.1038/s41598-017-10309-x.
38. Thanapati S, Ganu MA, Tripathy AS. Differential inhibitory and activating NK cell receptor levels and NK/NKT-like cell functionality in chronic and recovered stages of chikungunya. PLoS One. 2017;12(11):e0188342. doi:10.1371/journal.pone.0188342.
39. Noda K, Kodama S, Umemoto S, Abe N, Hirano T, Suzuki M. Nasal vaccination with P6 outer membrane protein and alpha-galactosylceramide induces nontypeable haemophilus influenzae-specific protective immunity associated with NKT cell activation and dendritic cell expansion in nasopharynx. Vaccine. 2010;28(31):5068–5074. doi:10.1016/j.vaccine.2010.05.005.
40. Klatka M, Grywalska E, Partyka M, Charytanowicz M, Kiszczak-Bochynska E, Rolinski J. Th17 and Treg cells in adolescents with Graves' disease. Impact of treatment with methimazole on these cell subsets. Autoimmunity. 2014;47(3):201–211. doi:10.3109/08916934.2013.879862.