Doctoral Thesis

Immunomodulatory Effects of CpG and Other Oligonucleotides

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Introduction

Vaccination methods are being developed rapidly nowadays because it became clear that vaccines are effective tools for the prevention and treatment of not only infectious diseases, but also tumors and allergy. In the course of immunization, it is important to evoke an isotype profile that is appropriate against the disease in question. This can be modulated by the vaccination strategy. Targeting of antigens to different immune receptors and the utilization inherent regulatory mechanisms of the immune system offer a tool for influencing or skewing the induced antibody or cellular responses. Receptor ligands can be used as tools to mediate activatory or - on the contrary - inhibitory signals in the context of the antigen with which they are coadministered with. An interesting example is the application of the ligands of pattern recognition receptors as vaccine adjuvants, like CpG oligodeoxynucleotides (CpG ODN).

Nucleic acids have been shown to be particularly potent molecular triggers of the innate immune response that not only provide a quick response against pathogens but also have a role in shaping the adaptive immune response. CpG oligonucleotides, that are responsible for the immune stimulating effect of bacterial DNA, contain non-methylated CG dinucleotides with specific flanking bases that were shown to activate Toll-like receptor 9 (TLR9). The nature of the DNA backbone (phosphodiesther or phosphorothioate), the sequence of the flanking bases and number of CG pairs in the sequence have a strong impact on the biological effects of CpG ODN. The phosphorothioate backbone modification endows ODN not only with nuclease resistance but also with other important properties like more facile cellular uptake and increased bioavailability. Due to the strong activating effect and stability of PS-ODN they became the preferred tools to elicit and study TLR9-mediated immune effects. In this work, our goal was to contribute to a better understanding of the functions of CpG and other synthetic ODN in the development of an immune response.

Aims

Our main goal was to gain knowledge concerning the effects of specific oligonucleotides on the developed immune response via differential actions on cells of the immune system. These oligonucleotides are well characterized from the perspective of APC activation through TLR9, but other mechanisms of action has been reported recently in different cell types. As these types of oligonucleotides are applied in modern vaccines as adjuvants, it is indispensable to define the full spectrum of the possible mechanisms of action of these molecules.

We studied CpG-mediated antigen uptake, immune response modulation and the role of T cell costimulation by different ODN in shaping B cell fate. We also looked for new methods for making the the analysis of the samples more informative, to gain more complex biological information from a single sample.

Our aims in detail were the following:

1. Characterization of the uptake of ODN complexes by APC and T cells.
2. Description of the effects of the co-administration of antigen-conjugated and free CpG as adjuvants on the immunogenicity of a model antigen in mice.
3. Comparison of the effects exerted by activating, control and inhibitory oligonucleotides on ODN and antigen uptake, cellular activation and differentiation of B cells into antibody secreting cells (ASC).
4. Examination of the requirement of cognate interactions between T and B cells for non-CpG modulation of T cell-dependent antibody production and isotype switching of B cells.
5. Development of novel multiplex methods that allow more efficient investigation of the humoral immune response utilizing the instrumentation of protein microarrays.

Methods

- Isolation, culturing and *in vitro* activation of murine spleen, lymph node and bone marrow cells and cell lines, differentiation of bone marrow-derived dendritic cells (BMDC)
- Flow cytometry (measurement of cell activation, proliferation, uptake of streptavidin (SA) complexes)
- Magnetic-activated cell sorting (MACS)
- Quantitative real-time PCR (measurement of TLR9 expression)
- Subcutaneous immunization of mice
- ELISA (Measurement of Ig secretion)
- Reverse Protein Microarray (Measurement of Ig secretion)
- ELISPOT (Detection of Ig-producing B cells)
- Fluorescent ELISPOT on nitrocellulose slides (simultaneous detection of different Ig-producing B cells)

Results and conclusions / I.

Cellular binding and activatory properties of CpG oligonucleotides *in vitro*.

- We have shown that SA-CpG complexes associate to dying, but not to living cells on ice. This implies that living cells does not bear a significant amount of ODN receptors on their surface, otherwise they would have bound SA-CpG complexes. Weak or missing anti-SA labeling also implies this.
- We have found that conjugation to CpG enhances cellular association and uptake of the antigen either by APC (B cells or BMDC) or by T cells after 24 h of incubation.
- Biotinylation and conjugation of CpG to SA does not influence the activatory potential of CpG ODN significantly as both free and antigen-conjugated CpG activate APC.

Results and conclusions / II.

There is a competition between free and antigen-conjugated oligonucleotides that adversely effects their immunogenicity.

- Free CpG competes with CpG-antigen conjugates for uptake by BMDC and T cells but shows weak or no competition in the case of B cells. The fact that saturation is more difficult to achieve in B cells can be explained by the much higher TLR9 expression of both untreated and activated B cells compared to BMDC and T cells.
- Although free CTRL does not activate APC it has a similar competition profile to CpG. The inhibitory INH ODN competes with SA-CpG but with less efficiency than CTRL or CpG ODN. It is possible that the different structure of the INH ODN results in this difference.
- Conjugation of the model antigen, SA to CpG, but not to the other two types of ODN (CTRL and INH), elevates the level of antigen-specific antibodies. At the dosage we used, only antigen-conjugated but not free CpG had statistically significant immune response enhancing effect.
- Using a vaccine formulation in which half amount of the CpG was conjugated to SA, and half of it was in free form, we have found that free CpG adversely influences the

adjuvant effect of antigen-conjugated CpG. The low TLR9 expression of dendritic cells - that we found *in vitro* - may be the cause of this phenomenon and this result points to the importance of choosing an appropriate vaccine composition and formulation.

Results and conclusions / III.

Non-CpG oligonucleotides enhance early and late activation events and also modulate differentiation into antibody secreting cells (ASC) and T cell-dependent antibody production, when cognate interactions take place between antigen-specific T and B cells.

- Non-CpG ODN enhance the early activation marker (CD69) expression of both T and B cells when these engage in cognate interaction.
- The expression of MHCII did not reflect our observations with CD69, but proliferation of both T and B cells reflected the pattern of early activation marker expression.
- We have developed a fluorescent ELISPOT method on microarray slides to perform multiplex measurements to identify and quantify different B cell populations simultaneously. This method requires shorter incubation times than conventional ELISPOT and it has high sensitivity. We utilized this technique to gain information about the number of ASC following antigen presentation and ODN costimulation.
- We have shown that differentiation into ASC is modulated by non-CpG ODN treatment. Non-CpG ODN thus enhances antibody production and isotype switching during cognate B cell-T cell interactions.
- Comparing wild type and TCR transgenic mice, we have shown that cognate interaction is required for the modulation of T cell induced antibody production by non-CpG ODN.
- Different ODN induces the development of distinct ASC isotype patterns that may suggest a differential mechanism of action for CpG and other types of ODN (Figure 1). The ratio of T_H1/T_H2 isotype ASC in the SA-CpG and SA-INH immunized groups differs significantly from the SA group, whereas immunization with SA-CTRL does not result in the emergence of a significantly different ratio of ASC compared to the SA group.

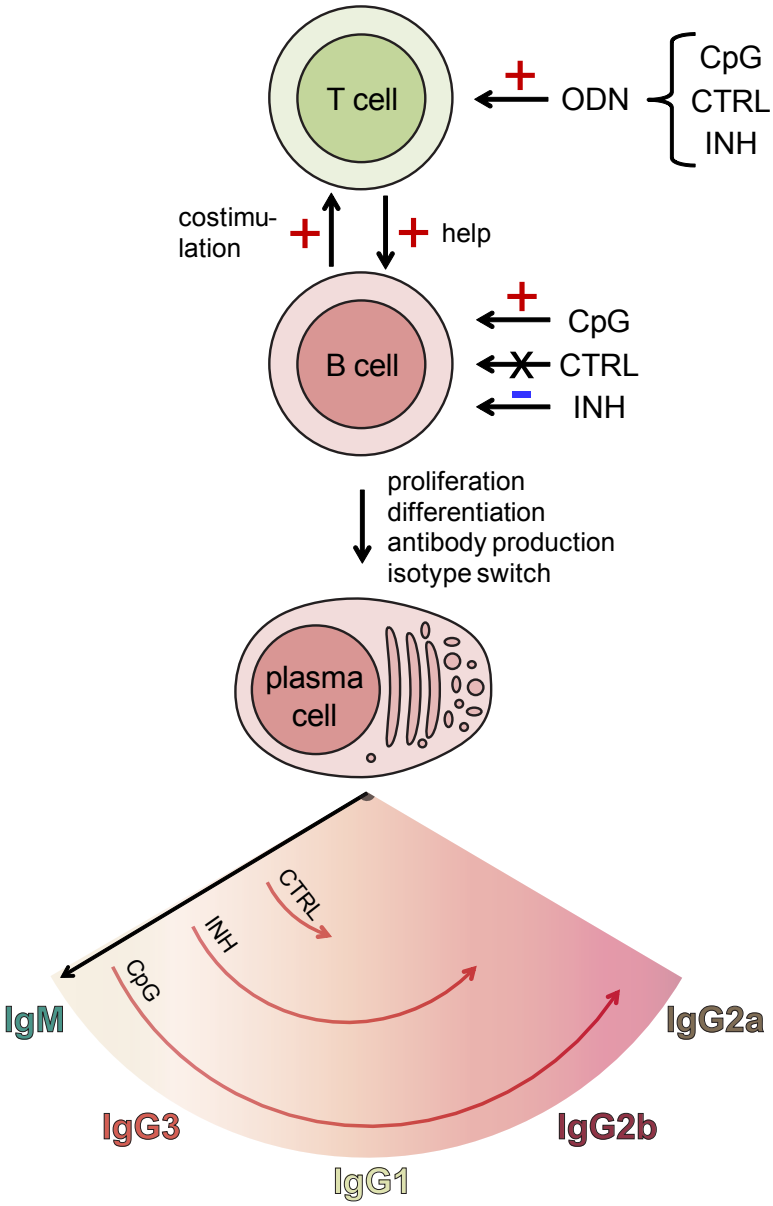

Figure 1: ***Illustration of the differential effects of distinct types of ODN on T and B cells during their cognate interaction.***

Different types of ODN costimulate T cells that are activated through TCR, while the same ODN have a different effect on B cells, depending on their nature. The outcome of the T cell-B cell interaction may be fine-tuned by their distinct responses to the given type of ODN and also depends on the subsequent other stimuli they receive.

Summary

Natural and synthetic nucleic acids are known to exert immunomodulatory properties. They are shown to modulate immune functions via several different pathways and various cell types, necessitating a complex interpretation of their effects. CpG motif containing oligodeoxynucleotides are widely studied as promising adjuvants in vaccines against a range of diseases including infection, cancer or allergy.

CpG ODN are used as adjuvants both in free from and as conjugates. Conjugation of antigen to CpG has been shown to potentiate the adjuvant effect via enhancing antigen uptake and promote danger signalling by the very same cell. In the present work, using SA as a model antigen, we described the effects of competition between antigen-coupled and free oligonucleotides. We have found that co-administration of these two forms influences the cellular uptake of the CpG-coupled antigen and adversely affects the immune responses following vaccination. Not only activating, but also control or inhibitory oligonucleotides have competitive effects *in vitro*.

While CpG oligonucleotides have been studied widely, further investigations would be neccessary to characterize the mechanism of action of other, non-CpG ODN. Therefore, we compared the effects of CpG ODN with those of non-CpG (control and an inhibitory) ODN in the context of cognate B cell - T cell interactions. We have found that non-CpG ODN exert significant immunomodulatory effects on early T cell and on late B cell activation events. Importantly, we have observed a synergism between antigen stimulus and non-CpG induced effects in T cells, that - following cognate T cell - B cell interactions - result in an enhanced IgG production by B cells and also affects their isotype profile.

Our findings support the argument that CpG-conjugated antigens are taken up predominantly in a receptor-mediated fashion and also point to the importance of TLR9 or other nucleic acid receptors in T cells that play a role in the development of an adaptive immune response. Taking everything into account, these observations are important for the optimal design of ODN containing vaccine formulations, as we have shown that antigen conjugated and free CpG should not be administered together and that non-CpG ODN may perform better as adjuvants when a strong antigen-independent immune activation, like that elicited by CpG ODN, would be undesirable.

Publications connected to the thesis

1. Melinda Herbáth, Krisztián Papp, Anna Erdei, József Prechl: "Non-CpG oligonucleotides exert adjuvant effects by enhancing cognate B cell - T cell interactions, leading to B cell Activation, Differentiation and Isotype Switching" Journal of Immunology Research (Article ID 340468, available online at http://downloads.hindawi.com/journals/jir/aa/340468.pdf)

2. Melinda Herbáth, Krisztián Papp, Andrea Balogh, János Matkó, József Prechl: "Exploiting fluorescence for multiplex immunoassays on protein microarrays" Methods and Applications in Fluorescence 2 032001. doi:10.1088/2050-6120/2/3/032001

3. Melinda Herbáth, Zsuzsanna Szekeres, Dorottya Kövesdi, Krisztián Papp, Anna Erdei, József Prechl: "Coadministration of antigen-conjugated and free CpG: Effects of in vitro and in vivo interactions in a murine model." Immunology Letters 2014 Feb 22. pii: S0165-2478(14)00029-7. doi: 10.1016/j.imlet.2014.02.007.

4. Zsuzsanna Szekeres, Melinda Herbáth, József Prechl: "Immune response modulation by targeted complexes based on streptavidin" Biochemistry Research Updates, Chapter 2 (pp. 49-85) Editor: Simon J. Baginski ISBN 978-1-61209-700-8

Other publications

1. Zsuzsanna Szekeres, Melinda Herbáth, Zoltán Szittner, Krisztián Papp, Anna Erdei, József Prechl: "Modulation of the humoral immune response by targeting CD40 and FcγRII/III; delivery of soluble but not particulate antigen to CD40 enhances antibody responses with a T_H1 bias" Molecular Immunology 2011. 49(1-2):155-62.

2. Zsuzsanna Szekeres, Melinda Herbáth, Adrienn Angyal, Zoltán Szittner, Viktor Virág, Péter Balogh, Anna Erdei, József Prechl: "Modulation of immune response by combined targeting of complement receptors and low-affinity Fcγ receptors" Immunology Letters 2010. 130(1-2):66-73.

Published abstracts

1. Zsuzsanna Szekeres, Melinda Herbáth, Zoltán Szittner, Péter Balogh, Anna Erdei, József Prechl: "Modulation of immune response by combined targeting of complement receptors and low affinity Fcγ receptors" *European Journal of Immunology, Vol. 39 Issue S1 (2009)*