Analysis of gene regulation and functional characteristics of the bovine neonatal Fc receptor using transgenic mice

Ph.D thesis

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2011

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Introduction

IgG is the most abundant antibody in the serum and plays a critical role in the maintenance of the long-lasting humoral immunity. The prolonged serum half-life and its importance in the maternal immunity makes IgG unique compared to other serum proteins. As a consequence of a receptor mediated process, IgG is protected from lysosomal degradation and has the longest half-life of all plasma proteins. The same receptor, the so called neonatal Fc receptor (FcRn), mediates the placental and intestinal IgG transport during the fetal and neonatal development thus providing the newborn with immunological protection in the weeks after birth. While we gain more and more knowledge about the function of the receptor there is not much information available about its expressional regulation. The clarification of its regulation and the investigation of its role in humoral immune response may provide important data to the better understanding of IgG homeostasis and may improve the efficiency of antibody production.

Aims / I.

The use of milk products derived from immunized cows as a passive immunization against intestinal pathogens is a long desired approach in human therapy. As cows produce colostrum with extremely high IgG concentration only for a few days, studies investigating the mechanism of IgG secretion into the colostrum have been initiated. Theoretically, via e.g. genetic modification, this IgG secretion can be elongated, which would result in high level pathogen-specific IgG in the milk and then could be used for intestinal protection in human therapy. FcRn plays - also in cattle - a pivotal role in the transport and homeostasis of IgG. Hence the aim of our group was to study the expressional regulation of this receptor in hope of its possible in vivo manipulation. As a first step, the genomic sequence and the promoter region of the bovine neonatal Fc receptor (bFcRn) heavy chain were analyzed. Our *in silico* studies suggested that the nuclear factor κB (NF-κB), which is a key member in the regulation of immunological processes, has specific binding sites on the promoter region of bFcRn. This hypothesis was than confirmed using *in vitro* studies, where the p65 subunit of human NF-κB

was able to activate the promoter of the bFcRn. Because human p65 was used for the bFcRn promoter studies we planned to set up a bovine-specific experimental system. Therefore, as a first step our group cloned and characterized the p65 subunit of the bovine NF-κB (bp65).

- The first aim of my studies in bFcRn gene regulation was to verify the functional properties of the cloned bp65 protein in a luciferase system and with immunohistochemistry.

After that we intended to analyze the effects of LPS mediated NF-κB activation on the bFcRn gene expression in an *in vitro*, as well as an *in vivo* system.

- The second aim was to establish a bovine endothelial cell based experimental system to analyze LPS mediated NF-κB activation on the bFcRn α-chain and β2-microglobulin gene expressions.
- The third aim was, while using LPS treatment, to assess the bFcRn gene expression in transgenic mice that carry multiple copies and overexpress the bFcRn.

Aims / II.

To keep the antibody titer high in polyclonal antibody producing animals, they are immunized repeatedly. During this process, the level of antigen-specific IgGs can exceed the normal titers, which can cause higher catabolic rates of all IgGs including the valuable antigen-specific ones. In our previous studies we showed that the clearance of injected ovalbumin-specific murine IgG was prolonged in transgenic (tg) mice expressing multiple copies of bFcRn, compared to wild type (wt) mice. We also showed that immunization of these tg animals with ovalbumin resulted in significantly higher levels of antigen-specific IgG as compared to wild type (wt) mice. Therefore our aim was to analyze the humoral immune response of these tg mice.

- We intended to study the immune response against T-independent and T-dependent (protein- and hapten-type) antigens, including weakly immunogenic ones, analyzing the antigen (Ag)-specific antibody titer and number of Ag-specific IgM and IgG producing B cells.

- We compared the relative affinity and relative quantity of the Ag-specific IgG molecules in wt and tg animals.
- We created bFcRn tg mice on BALB/c background (by back-crossing the original FVB/N strain onto BALB/c) and analyzed their immune response to prove that the elevated immune response found in FVB/N mice is strain independent and also because BALB/c mice are preferred in monoclonal antibody production.
- We wanted to compare the functional activity of tg and wt antibodies in a virus neutralization test after immunization of the mice with an influenza vaccine.

We observed that tg mice developed larger spleens during the immunization compared to wt mice.

- To clarify the reasons of the elevated humoral immune response and the larger spleen size in tg mice we intended to study the spleen cell composition from the immunized and unimmunized animals by cytofluorimetric analysis.

Results and conclusions / I.

- We verified that the p65 subunit of bovine NF-κB, cloned by our group, has the same cellular localization and functional characteristics as the human p65.

Transcription factors are highly conserved molecules, hence using them in different species is widely accepted. Our data show high similarity between the sequences and functions of the human and bovine p65, suggesting that the effects of human p65 on the bFcRn gene regulation can be accepted and considered as bovine-specific stimuli.

- To study the effects of LPS mediated NF-κB activation on bFcRn gene expression we established a bovine endothelial cell based experimental system. We demonstrated that LPS treatment activates the p65 subunit of the NF-κB transcription factor and upregulates the gene expression of the bFcRn α-chain and β2-microglobulin in these cells.

- We observed a quick increase in the bFcRn gene expression in transgenic mice overexpressing bFcRn after LPS treatment, and proved that this was not the result of the change of the spleen cell composition.

Besides of endothelial cells, also neutrophil granulocytes, macrophages and dendritic cells express bovine FcRn in these tg mice. It is likely that the increase in bFcRn expression in the spleen after *in vivo* LPS treatment resulted from the transcriptional upregulation in some of its cell populations. All these data suggest that a transcription mechanism, which is highly important in inflammatory processes or in infections, induces bFcRn upregulation contributing to the regulation of the immune response during infections and inflammation.

Results and conclusions / II.

- We immunized wt and bFcRn tg mice with protein- and hapten-type, thymus-dependent antigens and found significantly higher Ag-specific IgG and IgM titers in tg mice compared to wt controls. We also demonstrated that thymus-independent antigens did not induce elevated Ag-specific IgM production neither in wt nor in tg animals.
- We showed that the absolute and relative numbers of Ag-specific IgG and IgM producer B cells are higher in the spleen of tg mice compared to wt controls.
- We demonstrated that the affinity of Ag-specific IgGs was at least as good in tg mice as in the wt controls, which suggests that the affinity maturation is effective in both the tg and wt animals. Furthermore, we showed that the sera of the tg mice contain higher titers of high affinity Ag-specific antibodies as compared to the wt animals.
- We found a higher functional activity of virus-specific IgG antibodies in the sera of tg mice compared to wt controls in a virus neutralization test after immunization with an influenza vaccine.
- We observed that tg mice have larger spleens and contain proportionally more cells compared to wt mice after immunization. We analyzed the cell composition of the spleens and showed that proportionally more plasma cells, neutrophil granulocytes and dendritic cells are present in the spleen of tg mice after immunization compared to those of wt animals.

- We established another bFcRn tg mice strain with BALB/c genetic background and repeated the analysis of the immune response in these animals. Data showed similarly augmented immune response in the BALB/c tg mice as it was detected in FVB/N tg animals, confirming that the elevated humoral immune response induced by the overexpressed bFcRn is independent of the mouse genetic background.
- We also demonstrated that while weakly immunogenic antigens induced de minimis amount of antibodies in wt mice, bFcRn tg animals mounted a robust humoral immune response with high titers of Ag-specific IgG and IgM.

The higher Ag-specific IgM levels and the higher number of Ag-specific B cells found in bFcRn tg mice after immunization, suggest that the overexpression of bFcRn results not only in the prolonged IgG half-life, but also facilitates the clonal expansion of B cells in the spleen. The higher amount of IgG may form more immune complexes (IC) after the booster immunization in the tg animals, so we hypothesize that the elevated humoral immune response detected in tg mice may be due to the higher amount of ICs, which are able to activate more memory and more naïve B cells as well. The role of FcRn in the enhanced humoral immune response denotes a basic novelty concerning the function of this receptor. The enhanced humoral immune response with normal affinity maturation in bFcRn overexpressing tg mice offer major advantages in monoclonal and polyclonal antibody production, especially in the case of antibodies against weakly immunogenic antigens that otherwise would be difficult or even impossible to make.

Methods

- Cell transfection
- Immunohistochemistry
- Fluorescent microscopy
- PCR, Real-Time PCR
- Cytofluorimetry
- ELISA
- ELISPOT
- QCM (Quartz crystal microbalance)

Publications related to the Ph.D thesis

1. Márton Doleschall, Balázs Mayer, Judit Cervenak, László Cervenak, Imre Kacskovics: **"Cloning, expression and characterization of the bovine p65 subunit of NFkappaB"** Developmental and Comparative Immunology 31 (2007) 945–961 (IF: 3.29)

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3. Judit Cervenak, Balázs Bender, Zita Schneider, Melinda Magna, Bogdan V. Carstea, Károly Liliom, Anna Erdei, Zsuzsanna Bősze, Imre Kacskovics: **"FcRn overexpression boosts humoral immune response in transgenic mice"** Journal of Immunology, 2011Jan 15;186(2):959-68 (IF: 5.745)

4. Zita Schneider, Judit Cervenak, Mária Baranyi, Krisztián Papp, József Prechl, Glória László, Anna Erdei, Imre Kacskovics: **"Transgenic expression of bovine neonatal Fc receptor in mice boosts immune response and improves hybridoma production efficiency without any sign of autoimmunity"** Immunology Letters, 2011 Febr 137:62-69 (IF: 2.511)

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6. Kacskovics, I., Cervenak, J., Erdei, A., Goldsby, R.A., and Butler, J.E.: **"Recent Advances Using FcRn Overexpression in Transgenic Animals to Overcome Impediments of Standard Antibody Technologies"** MAbs, 2011 5: 431-439 (review)

7. Judit Cervenak, Márton Doleschall, Balázs Bender, Zoltán Doleschall, Balázs Mayer, Y. Zhao, Zsuzsanna Bősze, Lennart Hammarström, Imre Kacskovics: **„NF-κB stimulates the bovine FcRn expression”** manuscript in preparation

Patent related to the Ph.D thesis

Zs. Bősze, I. Kacskovics, J. Cervenak, L. Hiripi, B. Bender: „**Method using a transgenic animal with enhanced immune response” (EP2097444).** 2011. In European Patent Office 22.06.2011 Bulletin 2011/25. B. Eotvos Lorand University, and G. Agricultural Biotechnological Center, eds, European Patent Specification. 66.

Attending on international conferences

1. Mayer B., Doleschall M., Benkő S., Cervenak J., Ludányi K., Doleschall Z., Cervenak L., Rajnavölgyi É. and Kacskovics I. **“Human FcRn α-cain is differentially expressed in monocytes, macrophages and dendritic cells”** 16th European Immunology Congress, 2006, Paris, France

2. Doleschall M., Mayer B., Cervenak J., Zhao Y., Doleschall Z., Cervenak L., Hammarström L. and Kacskovics I. **“NFκB does not induce bovine β_2-microglobulin expression due to a deletion in its promoter”** 16th European Immunology Congress, 2006, Paris, France

3. Z. Schneider, J. Cervenak**,** M. Magna, B. Bender, A. Erdei, Zs. Bősze, I. Kacskovics: **“FcRn overexpression boost T-dependent humoral immune response”** 2nd European Congress of Immunology, 2009, Berlin, Germany

Attending on national conferences

1. Cervenak J., Bender B., Magna M., Schneider Z., Erdei A., Bősze Zs., Kacskovics I.: **„Az IgG-kötő FcRn fokozott expressziója jelentősen növeli az immunizálás hatékonyságát”** Magyar Immunológiai Társaság XXXVI. kongresszusa, 2007, Hajdúszoboszló; ***First place at the Sigma-Aldrich competition in the category of Fundamental Research in Immunology***

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3. J. Cervenak, M. Magna, B. Bender, Z. Schneider, A. Farkas, L. Hiripi, A. Erdei, Z. Bősze and I. Kacskovics: **“FcRn overexpression boosts humoral immune response”** 15th*efis*-EJI Symposium on Signals and signal processing in the immune system, 2009, Balatonőszöd

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